



THE ROLE of MANNOSE BINDING LECTIN (MBL) in PAEDIATRIC INFECTION

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ABSTRACT

Mannose-binding lectin (MBL) is a circulating pattern-recognition molecule that recognizes microbial carbohydrate motifs leading to complement activation and cell lysis. Mutations in MBL promoter and exon-1 result in reduced protein levels and in a number of studies appear to increase susceptibility to infection. This thesis explores the role of MBL in paediatric infection in two clinical settings.

The effect of MBL deficiency on susceptibility and progression of HIV-1 infection was investigated in one hundred and twenty eight children, aged 2-16 years. MBL deficiency appeared to be less frequent in this population than in published series of Caucasian or African children. This may be due to selective survival of children with wild type MBL genotypes as patients with severe disease, as assessed by low CD4+T cell counts, were more likely to have MBL variant alleles. In support of this hypothesis, MBL deficiency was less frequent in children classified as long term non- progressors (LTNP-s).

A second study explored the impact of MBL on susceptibility and severity of infection in preterm neonates. One hundred sixty six preterm neonates were genotyped for MBL mutations by polymerase chain reaction (PCR) and heteroduplex analyses. Serum MBL levels were measured by ELISA. Comparison of genotypes (A= wild type, 0=variant alleles) and protein levels between groups was performed using Chi-square, Mann-Whitney or Kruskal-Wallis test. Low MBL levels were observed in premature neonates, particularly in the first week of life ($p=0.001$). MBL deficiency was associated with an increased risk of sepsis in VLBW neonates ($\leq 1500\text{g}$), ($p=0.01$).

The studies described in this thesis provide support for MBL having a role in susceptibility to and severity of infection in children.

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It is a great pleasure to have a profession where not a day passes that I don't learn something new. Undertaking an M Phil has opened my eyes to the fact that I didn't really understand many of occurrences I observed and attempted to manipulate. It has also made me aware as a doctor, that many of things we credit ourselves with achieving when managing patients are probably being achieved by the homeostatic mechanisms that exist within our bodies. Despite the glimpse into the extraordinary amount of new information that is being discovered every day in science, the art of medicine is fundamental to its successful practice and being a truly great doctor.

Agnieszka B. Dzwonek January 2007.

DECLARATION

The work presented in this thesis was performed by myself. I would like to acknowledge the importance of specific contribution of the following Departments and Institutions.

The work presented in chapter 3 was carried out at Great Ormond Street Hospital, HIV Family Clinic. I have set up the HIV study described in Chapter 3. I recruited all the children, collected blood samples, performed the laboratory and statistical analysis and collected the clinical data.

The work presented in chapter 4 was carried out as a part of collaborative study with the departments of Immunology and Neonatal Intensive Care Units at the Polish Mother Memorial Hospital-Research Institute and University College London Hospitals, NHS Trust. Blood samples and clinical data were collected jointly. All laboratory analysis, statistical analysis, and discussion are solely the work of the author.

I personally conducted all the laboratory experiments described in this thesis. In addition I have analysed and interpreted results of all studies described in this thesis. Statistical support was received by Dr Angie Wade from Department of Statistics and Rodolphe Thiebaunt at the ICH/UCL. Thank you last but not least, my principal supervisor, Professor Nigel Klein, who has a significant intellectual contribution to this work, helped me with interpretation of the data and supervised the writing of this thesis.

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Abbreviations

| | |
|-------|--|
| AIDS | Acquired Immunodeficiency Syndrome |
| ARIs | Acute respiratory tract infections |
| ART | Antiretroviral therapy |
| ARTN | Aniteroviral therapy Naive |
| CDC | Centers for Disease Control and Prevention |
| CF | Cystic fibrosis |
| CoNS | Coagulase negative <i>Staphylococci</i> |
| CSF | Cerebrospinal fluid |
| CRD | Carbohydrate recognition domain |
| CRP | C-reactive protein |
| DCs | Dendritic cells |
| DKO | Double knock-out |
| DNA | Deoxyribonucleic acid |
| dNTP | Deoxynucleotide triphosphates |
| ELISA | Enzyme-linked immunosorbent assay |
| ELBW | Extremely low birth weight |
| HAART | Highly Active Antiretroviral Therapy |
| HIV | Human Immunodeficiency Virus |
| HSV | Human Simplex Virus |
| ICAM | Intracellular adhesion molecule |
| IQR | interquartile range |
| LPS | Lipopolysaccharide |

| | |
|-------------------|---|
| LTNP | Long-Term Non Progressors |
| SIRS | Systemic Inflammatory Response Syndrome |
| SNPs | Single nucleotide polymorphisms |
| MAC | membrane attack complex |
| MASP | Mannose-binding lectin (MBL) associated serine protease |
| MBL | Mannose-binding lectin |
| MgCl ₂ | Magnesium chloride |
| min | minute |
| μ | micro |
| ml | millilitre |
| NK | natural killer |
| ng | nanogram |
| PAMP | Pathogen-associated molecular patterns |
| PCR | Polymerase chain reaction |
| PI | Primary isolates |
| PG | Peptidoglycan |
| PRR | Pattern Recognition receptors |
| RNA | Ribonucleic acid |
| RT | Room temperature |
| Rpm | Revolutions per minute |
| rhMBL | recombinant human MBL |
| sMAP | small MBL-associated protein |
| SAP | Serum Amyloid protein |

| | |
|------------|----------------------------------|
| SP-A and D | Surfactant protein A and D |
| TLR | Toll-like receptors |
| TNF | Tumor Necrosis Factor |
| UBC | Umbilical cord blood |
| UHG | Universal Heteroduplex Generator |
| VLBW | Very low birth weight |
| WHO | World Health Organization |
| WT | Wild Type |

CHAPTER 1

INTRODUCTION AND THESIS AIMS

1.1 MANNOSE BINDING LECTIN and INNATE IMMUNITY

Mannose-Binding Lectin (MBL) is a part of the humoral innate immune system. It is a pattern-recognition molecule able to detect a wide range of microbial and altered-self targets and recruit a number of host immune effector systems to clear those targets (Turner 1996). Profound genetic deficiency of MBL is common (less than 400 ng/ml) in ~7-10% of the Caucasian population and appears to predispose individuals to a broad range of infectious diseases (Turner 1998; Turner and Hamvas 2000). Low-producing *MBL-2* variants and low MBL levels are associated with increased susceptibility to and severity of a variety of infective illnesses, particularly when immunity is already compromised- for example, in infants and young children, patients with cystic fibrosis, and after chemotherapy and transplantation. The aim of studies presented in this thesis was to determine the potential role of MBL in susceptibility to bacterial infection in preterm neonates, and in Human Immunodeficiency Virus-1 (HIV-1) infection in children.

The innate immune system is our first line of defence against pathogenic micro-organisms. All components of innate immunity are present prior to exposure to micro-organisms. Our other major defence mechanism is the specific adaptive immune system which can take several days to mount an adequate response. In preterm neonates, immature adaptive response and the cellular effectors of innate response such as neutrophils, macrophages, dendritic cells and natural killer cells maybe reduced in number or may operate sub-optimally.

The innate immune system is being increasingly recognized as a central defence mechanism against infection (Janeway and Medzhitov, 2002). The early phases of the host response to infection depend on innate immunity in which a variety of innate resistance mechanisms recognize and respond to the presence of a pathogen. The innate immunity system composes cellular and humoral components which recognise the general features of microbes in order to clear these potentially damaging agents from body (Medzhitov and Janeway, 1997; Hoffmann *et al.*, 1999). In contrast to the acquired immune system this does not require prior exposure to the infectious agent. One of the characteristic features of the innate immune system is microbial recognition. The specificity of the recognition systems employed is targeted against highly conserved structures common to large groups of microorganisms. This is achieved through interactions between host-derived pattern recognition receptors (PRR) and pathogen associated molecular patterns (PAMP) on microbes, which are frequently the repeating sugar arrays expressed on microbial surfaces (Janeway, 1989).

PRRs can be presented on the cell surface, in intracellular compartments, or secreted into the bloodstream and tissue fluids (Medzhitov and Janeway, 1997; Takeda and Akira, 2004). They are expressed on most effector cells of the immune system and, of particular relevance to innate immunity, is their presence on mucosal epithelia, macrophages, dendritic cells (DC) and neutrophils (Takeda and Akira, 2004).

The cell-surface PRRs include the well characterized membrane-bound toll-like receptors (TLR). The macrophage mannose receptor, is predominantly expressed by macrophages, but also by other cells such as dendritic cells, hepatic and lymphatic

endothelia. It functions as a PRR that mediate phagocytosis of microorganisms in the absence of opsonins, indicating that mannose receptor mediated lectin- phagocytosis maybe particularly important in anatomical spaces where opsonins maybe limited. Like MBL, macrophage mannose receptor is a member of the C-type lectin family and is closely related to Dectin-1 (Dendritic cell associated lectin-1)-an NK-like C-type lectin-like, DC-SIGN (dendritic cell-specific ICAM-3 grabbing non-integrin) and DC-SIGNR (DC-SIGN-related) expressed on dendritic cells.

The main function of the macrophage mannose receptor is pathogen recognition and phagocytosis of microbial pathogens, delivery to lysosomal compartment where there are destroyed by lysosomal enzymes (Fraser *et al.*, 1998). Other functions of MMR are clearance of endogenous ligands such as serum glycoproteins, lutropin, myeloperoxidase and thyroglobulin, and antigen presentation.

MBL, SP-A and D (surfactant protein A and D), C-reactive protein (CRP) and SAP (serum amyloid protein) are secreted pattern recognition molecules produced by the liver during the acute phase response in the early stages of an infection (Schwalbe *et al.*, 1992; Fraser *et al.*, 1998). Secreted PRRs function as opsonins by binding to microbial cell-walls and targeting them for recognition by the complement system and phagocytes (Medzhitov and Janeway 2000; Turner, 2003).

PAMPs include conserved structures such as Lipopolysaccharide (LPS), Peptidoglycan (PG) and flagellin which are found on most microorganisms but not on host cells thus

allowing the immune system to distinguish “self” from “non-self” (Janeway, 1989; Medzhitov and Janeway, 2000; Takeda and Akira, 2004).

The role of MBL in susceptibility to infection is the subject of this thesis. For this purpose the structure and function of this critical secreted pattern recognition molecule (PRR) is discussed in greater detail below.

1.2 THE COMPLEMENT SYSTEM

Complement is an important system in the humoral innate response and is a major part of the human immune system that is responsible for defence against foreign particles and the clearance of immune complexes. Its actions “complement” the role of antibodies in the destruction of invading microorganisms. Complement protects against infection in three ways, 1) it generates large numbers of activated proteins which bind to pathogens, opsonising them for engulfment by phagocytes, 2) small fragments of some complement proteins act as chemoattractants to recruit more phagocytes to site of infection and 3) terminal components damage certain bacteria by creating pores in the bacteria through the generation of the membrane attack complex (MAC).

The complement system was first described in the 1890s as a heat labile protein in serum that “complemented” heat stable antibodies in the killing of bacteria. It is now known to be a highly developed host-defence system involved both in innate and antibody-mediated immunity (Walport, 2001a; Walport, 2001b). It consists of more than 30

plasma and cell surface protein components involved in a cascade of reactions. The formation of antibody and antigen complex induces conformational change in the Fc portion of the antibody molecule that exposes a binding site for C1q. Binding of C1q to Fc binding sites induces a conformational change in C1r that converts C1r to an active serine protease enzyme. C1r then cleaves C1s to its active form, which in turn activates C4 and C2 components. The resulting C4b2a complex is a C3 convertase, which converts the C3 proenzyme into the active enzyme C3a. This cascade leads to the cleavage of C3, a major (~concentration 1mg/ml) serum protein. The above sequence of events is referred as the activation pathway (shown diagrammatically in Fig 1.1).

It is followed by the lytic pathway, during which the MAC is formed. As shown at figure 1.1 there are three distinct complement pathways of activation which depend on different molecules for their initiation but converge to generate the same set of effector molecules:

- 1) The Classical Pathway
- 2) Alternative Pathway
- 3) Lectin Pathway.

Figure 1.1 The complement system (taken from Presanis *et al.*, 2003)

These three pathways all generate C3 convertase which leads to the generation of C3. The splitting of C3 produces a small C3a fragment and the larger C3b. Many interactions are possible at this point, but to progress towards cell lysis the classical and alternative pathway complexes, C4b2a and C3bBb, must covalently bind C3b. This results in new enzyme specificity-the C5 convertase. C3b forms a covalent dimer with the surface bound C4b or C3b of the C3 convertase. C5 binds to these dimers and is catalytically cleaved by the complex releasing the C5a anaphylatoxin and C5b, the first member of the terminal or lytic pathway. The alternative pathway is initiated by the covalent binding of a small amount of C3 to hydroxyl or amine groups on the cell-

surface molecules of microorganisms. This pathway also functions to amplify the activation of C3 (amplification loop). The lytic pathway essentially involves non-covalent binding of C5b to C6 and C7, producing a complex, C5b-7, which is hydrophobic in nature and can insert itself into lipid membrane. The binding of C8 further enhances this process and leads to the polymeric assembly of C9 within the membrane. This MAC, C5b-9, causes lesions in the lipid membrane and results in cell lysis.

Defects in complement system are associated with a wide range of disease processes including susceptibility to infection, modulation of inflammatory processes and defects in regulation (Walport 2001a; Walport 2001b). Clinical implications of complement pathways deficiencies are summarized in Figure 1.2.

The clinical history of patients with classical pathway deficiencies varies slightly from other complement-deficient patients. In the small number of patients studied, patients with classical pathway deficiencies (i.e., deficiency of C1qrs, C2, or C4) are similar in presentation to patients with primary immunoglobulin deficiencies. For example, patients tend to have frequent sino-pulmonary infections with organisms such as *Streptococcus pneumoniae* or *Hemophilus influenzae*^e. More commonly, these patients develop autoimmune syndromes. One of the primary clinical manifestations of patients with deficiencies of classical pathway components is immune complex disease.

The C3 component occupies an important place at the junction of both the classic and alternative pathways. As such, C3 deficiency results in severe opsonization dysfunction.

C3 deficiency also causes deficient leukocyte chemotaxis because of decreased C3a concentrations and decreased bactericidal killing secondary to decreased formation of membrane-attack complex (MAC). Patients with C3 deficiency develop severe episodes of recurrent pneumonia, meningitis, peritonitis, or sepsis. The most common pathogens are *Streptococcus pneumoniae*, *N meningitidis*, *Haemophilus influenzae*, and *Staphylococcus aureus*. In addition to opsonization problems, C3 deficiency also impairs adequate clearance of circulating immune complexes, and 79% of patients with C3 deficiency develop some form of collagen vascular disease. The inherited deficiency of the MAC causes bacterial infection, particularly *Neisserial* infections.

Figure 1.2 The clinical implications of complement pathways deficiencies (from eMedicine)

Deficiency states may be acquired or inherited. Acquired defects are relatively common, may be acute or chronic and are frequently reversible with treatment of the disease responsible for the defect. Infection is an uncommon complication of acutely acquired defects but is a well recognized component of chronic deficiency states. Acquired defects are classified according to the mechanism responsible for the defect: consumptive, synthetic, or catabolic. Immune complex disease, vasculitis, and C3 nephritic factor are examples of conditions in which complement activity is low as a result of consumption. Decreased synthesis is most commonly observed in patients with severe liver diseases. Accelerated catabolism of complement proteins occurs in patients with protein losing enteropathies or the nephritic syndrome. Hereditary complement deficiencies are uncommon (about 0.03% prevalence) in the general population. Homozygous C2 deficiency appears to be the most common inherited defects.

1.2.1 The lectin pathway of complement activation

The lectin pathway was discovered in 1989 (Super *et al.*, 1989). This pathway involves carbohydrate recognition on microbial surfaces by pattern recognition molecule, such as MBL, independently of specific antibody. MBL is an important component of innate immunity and is a central recognition molecule of the lectin pathway of complement. MBL is a member of the Collectin family of proteins which are characterized by the presence of a collagenous region and C-type lectin carbohydrate recognition domain (CRD), (Malhorta *et al.*, 1994; Turner, 2003; van de Wetering *et al.*, 2004). It is an acute phase protein of hepatic origin. MBL binds to repeating sugar arrays on microbial surfaces including Gram-positive and Gram-negative bacteria, to fungal species, some

viruses and also to protozoa and other parasites (Table 1.1; Kilpatrick 2002; Jack and Turner, 2003, Shang *et al.*, 2005).

Table1.1 MBL binding to micro-organisms. Data from various studies have been included (copied from Kilpatrick 2002).

MBL functions as an opsonin and its biological effect is mediated by direct killing *via* complement through MAC or by promoting phagocytosis either by the lectin pathway of complement or by direct binding to one or more cell surface receptors (Turner 1996; Neth *et al.*, 2000; Gadjeva *et al.*, 2004b).

The lectin pathway also comprises three MBL associated serine proteases, MASP-1, -2, -3 and MAP19/ sMAP, a small MBL associated protein with no serine protease activity (Stover *et al.*, 1999; Dahl *et al.*, 2001). The MASPs are homologous to the C1q-associated proteases, C1r and C1s. MBL therefore activates complement by a mechanism very similar to C1q, and engages the opsonic activity of complement to clear micro-organisms. MASP-2 is a homologue of C1s of the classical complement pathway and is responsible for the activation of C4 and C2. When MBL associated with MASP-2 binds to sugar arrays on the surface of microbes, the MBL-MASP-2 pro-enzyme is activated and sequentially cleaves C4 and C2 thereby generating the C4bC2a complex, a potent C3 convertase (Thiel *et al.*, 1997; Matsushita and Fujita, 2001). C4b2a cleaves C3 and generates multiple C3b fragments which bind covalently to the surface of microorganisms and acts as a key opsonin. These fragments are recognized by the CR1 (CD35) receptor of phagocytes. Some C3b is converted to iC3b which is recognised by CR3 receptors. The C3b fragment also facilitates activity of C5 convertase. Cleavage of C5 produces the anaphylatoxin C5a, and the C5b fragment initiates formation of the MAC causing direct lysis of the inciting pathogen (Figure 1.2).

MBL isolated from serum is apparently only associated with MASP-1 and MASP-2 and not C1r or C1s (Thiel *et al.*, 1997). It has been reported that MASP-1 is able to cleave

C3 directly (Matsushita *et al.*, 2000; Dahl *et al.*, 2001; Rossi *et al.*, 2001). Work with recombinant and purified native MASP-1 shows that direct C3 cleavage by this protease is very slow and may not be biologically significant (Rossi *et al.*, 2001). MASP-1 does not appear to have a role in activating MASP-2, and has very limited or negligible activity towards other complement proteins. Recently, a third MASP (MASP-3), which is generated by alternative splicing of MASP-1, was reported to be associated with MBL but its function and also that of MAP19 is presently unknown, although MASP-3 may compete with MASP-2 for binding to MBL (Dahl *et al.*, 2001).

The lectin pathway is also known to be activated by human serum ficolins: L-ficolin and H-ficolin (Matsushita and Fujita, 2002; Holmskov *et al.*, 2003; Lynch *et al.*, 2004) and by M-ficolin, a secretory protein (Liu *et al.*, 2005). The ficolins comprise a collagen- and fibrinogen like domains (Matsushita and Fujita, 2001; Fujita, 2002), (Figure 1.3). They also bind to bacteria in association with MASPs and small MBL-associated protein (sMAP) and are believed to have complementary and overlapping microbial specificities with MBL (Matsushita and Fujita, 2002).

1.3 MBL SYNTHESIS, STRUCTURE and FUNCTION

MBL was first isolated and cloned from hepatocytes, and the liver is considered to be the principal site of MBL synthesis (Summerfield and Taylor, 1986). Subsequent studies confirmed that MBL is produced by hepatocytes and secreted into the bloodstream (Ezekowitz *et al.*, 1988; Taylor *et al.*, 1989; Sastry *et al.*, 1989). MBL has also been

found in body fluids, including nasopharyngeal secretions, middle ear effusions, ascites, urine, cerebrospinal fluid (CSF), amniotic fluid and synovial fluid (Garred *et al.*, 1993; Terai *et al.*, 1993). Two studies have assessed MBL production in advanced liver disease (Ryley *et al.*, 1993; Homann *et al.*, 1995). The first found that in chronic liver disease, stainable liver MBL did not correlate with serum MBL levels (Ryley *et al.*, 1993). The second study showed that serum MBL was significantly elevated in patients with cirrhosis (Homann *et al.*, 1995). These observations may suggest that there is extra-hepatic contribution to serum MBL. Experimental data support the concept of non-hepatic MBL synthesis. Human monocytes and monocyte-derived dendritic cells are both capable of synthesizing MBL *in vitro* (Downing *et al.*, 2003). Several rodent studies have also demonstrated extra-hepatic expression of MBL, with the sites responsible including small intestine, kidney, as well as lymphoid and myeloid cells (Morio *et al.*, 1997; Uemura *et al.*, 2002; Wagner *et al.*, 2003). Studies on MBL secretion from rat hepatocytes suggest that the protein is synthesised in the endoplasmic reticulum before transportation to the golgi apparatus prior to secretion. The half-life ($T_{1/2}$) of secretion is 4 hours, which is long compared to other hepatocyte secretory proteins (e.g. $T_{1/2}$ = 1 hour for albumin). Once in circulation, serum MBL turnover is relatively fast with a $T_{1/2}$ of 1.6 days when compared with other serum proteins (~3 days).

The human MBL monomer is 32kD in size and composed of 208 amino-acids (Kawasaki *et al.*, 1983; Summerfield and Taylor, 1986; Taylor *et al.*, 1989; Sastry *et al.*, 1989). MBL peptide contains an N-terminal region of 21 amino acids followed by the collagenous region which consists of 19 glycine-X-Y repeats (where X and Y can be any amino-acid) with an interruption of this pattern at repeat 8 (Taylor *et al.*, 1989, Sastry *et al.*

al., 1989). In human MBL, a neck region (residues 88-113) connects to the calcium dependant CRD which recognizes repetitive oligosaccharide moieties present on a wide array of pathogens (Figure 1.3 a; Sheriff *et al.*, 1994).



Figure 1.3 Diagram showing the single MBL peptide (A), basic structural sub-unit (B), and oligomeric structure of MBL (the hexameric form) (C), (modified from Fujita *et al.*, 2004).

The basic structural unit of MBL is a homotrimer of MBL peptides (sub-units) that self associate into a collagen-like triple helix (structural unit, Figure 1.3 b). This structural unit oligomerises into high order structures such as tetramers, pentamers and hexamers (Figure 1.3 c).

Functional MBL circulates as higher order multimers. The higher order structure allows high-affinity interaction between MBL lectin domains and microbial oligosaccharides, resulting in conformational change of the MBL multimer and activation of associated molecules, MASPs leading to complement activation.

The serum levels of the human MBL are regulated in serum so that any one person displays a phenotype of low, intermediate, or high levels. There is a close relationship between circulating MBL and distinct genotypes. A comprehensive study by Minchinton and colleagues showed the correlation between MBL genotype and protein levels (Figure 1.4), (Minchinton *et al.*, 2002). Humans exhibit a 1000-fold variation in circulating MBL from <0.01 to 5 µg/ml or more.

Genotype has the largest influence on circulating MBL levels but non-genetic influences are also important. MBL is constitutively expressed but serum levels can increase up to threefold during an acute phase response, after surgery or infection (Thiel *et al.*, 1992).

The gene encoding MBL, *MBL-2*, contains several common polymorphisms that influence transcription and disrupt assembly of the molecule, in particular distorting the collagenous helix and impairing polymerisation to higher order functional multimers

(Larsen *et al.*, 2004). Although high and low molecular-mass forms of MBL can be detected in serum, studies have shown that it is only higher order oligomers that are able to confer function such as binding to microorganisms and activation of complement (Larsen *et al.*, 2004).



Figure 1.4 Correlation between MBL levels and genotypes. Genotypes are in a simplified form, A/A indicates wild type excluding XA/XA; A/O indicates heterozygotes including XA/XA; O/O indicates homozygote mutants and compound heterozygote mutants (Minchinton *et al.*, 2002).

MBL gene transcription can be modulated by interleukin (IL-6) and dexamethasone as observed in human hepatoma cell lines (Arai *et al.*, 1993). IL-6, dexamethasone increased MBL gene expression in this cell line. Interestingly, IL-1 inhibited transcription whereas TNF- α and INF γ had no effect (Arai *et al.*, 1993).

Another study was able to detect low levels of MBL mRNA in the HepG2 cell line (Ezekowitz *et al.*, 1988). MBL levels may also be influenced by different hormones *in vivo* (Sorensen *et al.*, 2006). The recent study by Sorensen and co-workers suggested that growth hormone (GH), interleukin (IL-6) and thyroid hormones (T3 or T4) significantly increased MBL synthesis in a dose-manner (Sorensen *et al.*, 2006).

1.4 MBL GENE STRUCTURE

Human MBL is encoded by the *MBL-2* gene on chromosome 10q11.3 (Sastry *et al.*, 1989 and Taylor *et al.*, 1989). The intron / exon structure of the 7 kb human gene has four exons. These regions comprise an N-terminal cysteine-rich domain (exon 1), a collagen region (exons 1 and 2), followed by an α -helical neck region (exon 3). The C-terminal encodes for the carbohydrate recognition domain (exon 4) (Taylor *et al.*, 1989). A schematic representation of the MBL gene organisation is shown in Figure 1.5.



Figure 1.5 Human MBL 2 gene structure and the MBL mutations (modified from Larsen *et al.*, 2004).

At the *top* naturally occurring mutations MBL-B, -C, and -D are highlighted.

Below is the wild-type (WT MBL-A) protein sequence of the collagen region of MBL.

The various protein domains of the single polypeptide chain of MBL: the cysteine rich N-terminal, the collagenous region (snaking line), the α helical coiled-coil region, and the CRD are shown schematically.

Several studies have now shown that the level of MBL in blood is influenced by the presence of single nucleotide polymorphisms (SNPs) in the *MBL-2* gene (Madsen *et al.*, 1995; 1998). Three missense mutations in exon 1 at codons 52, 54, and 57 encode for variant alleles termed as D, B, and C, respectively, reflecting the order of their discovery (Lipscombe *et al.*, 1992; Turner 2003). The presence of any of these coding mutations is represented by “O”. By convention, a wild-type coding region is termed the “A” allele. A point mutation at base 230 of exon 1 of the *MBL-2* gene changes codon 54 (allele B) from GGC to GAC resulting in amino-acid substitution from a glycine to a much larger (in size) aspartic acid in the collagen region. Similarly, a point mutation in codon 57 (allele C) results in a glutamic acid residue instead of a conserved glycine (Lipscombe *et al.*, 1992). A third structural gene mutation in codon 52 (D allele) was described in 1994 (Madsen *et al.*, 1994). This change results in arginine being substituted by a cysteine residue. This mutation appears to have a less severe effect on the levels of circulating MBL when compared to allele B and C (Madsen *et al.*, 1994).

Studies of different ethnic groups indicate that the prevalent haplotypes that specify high, intermediate, low or undetectable levels of MBL in serum vary considerably (Lipscombe *et al.*, 1992; Madsen *et al.*, 1994; Madsen *et al.*, 1995; Lipscombe *et al.*, 1996; Mead *et al.*, 1997; Madsen *et al.*, 1998; Turner *et al.*, 2000(a); Minchinton *et al.*, 2002). *MBL-2* coding alleles associated with low blood levels are present in up to 40% of Caucasoids, with up to 8% having genotypes associated with profound reduction in circulating MBL levels. The frequencies of the coding mutations studied in various population groups are listed in Table 1.2.

Table 1.2 Frequency of exon-1 mutations found in the human *MBL* gene amongst different population groups (according to Lipscombe *et al.*, 1992).

The codon 54 mutation has been identified in a range of European and Asian populations at a frequency of 0.13-0.16 (Lipscombe *et al.*, 1992; Garred *et al.*, 1992; Madsen *et al.*, 1994) and the codon 57 mutation occurs in most Sub-Saharan African populations at even higher frequencies: 0.23-0.29 (Lipscombe *et al.*, 1992 and 1996; Garred *et al.*, 1992; Madsen *et al.*, 1994). The codon 52 mutation occurs at much lower frequencies (0.06 or less) both in African and non- African populations.

Serum MBL levels, however, vary substantially within each coding genotype. Additional polymorphisms in the promoter and 5'-untranslated regions of the *MBL-2* gene have a modulatory effect on basal MBL protein levels (Madsen *et al.*, 1995; Garred *et al.*, 2003a). Single nucleotide substitutions are present in the promoter region at

position -550 (G→C, alleles “H” and “L”); position -221 (G →C, alleles “X” and “Y”); and at nucleotide +4 of the 5'-untranslated region (C→T, alleles “P” and “Q”). The -550 (H/ L), -221 (X/ Y), +4 (P/ Q) promoter region polymorphisms occur in one of the following linkage haplotypes HYP, LYQ, LYP or LXP; superimposing very high, high, medium and low levels onto the exon-1 genotype respectively (Madsen *et al.*, 1995; Madsen *et al.*, 1998). One of these variants (X/ Y) profoundly influences expression of the MBL. There is also linkage disequilibrium between the structural and promoter mutations such that a B allele is always linked to an LYP promoter genotype, a C allele to an LYQ promoter genotype and a D allele to an HYP promoter genotype (Lipscombe *et al.*, 1996). The wild-type genotype (A/A) is found in association with four promoter haplotypes.

1.5 MBL and HOST DEFENCE

1.5.1 The role of MBL in opsonization and phagocytosis

MBL may also interact directly with cell surface receptors and thereby promote opsonophagocytosis and modify cellular activation. This property was first reported by Kuhlman *et al.*, (1989) in a study of MBL coated *Salmonella enterica* serovar *Montevideo* organisms. MBL opsonized *Salmonella enterica* directly without the involvement of the complement system (Kuhlman *et al.*, 1989). In another studies, the phagocytosis of *Cryptococcus neoformans* or *Mycobacterium avium* was significantly enhanced by recombinant MBL (Levitz *et al.*, 1993; Polotsky *et al.*, 1997). Subsequently, a number of putative MBL binding proteins/ receptors have been

proposed including cC1qR/ calreticulin (Malhotra *et al.*, 1994; Stuart *et al.*, 1997), C1qRp/ CD93 (Tenner *et al.*, 1995; Steinberger *et al.*, 2002) and CR1/ CD35 (Ghiran *et al.*, 2000). The recent study by Neth and co-workers showed that MBL-MASP complexes can promote opsonophagocytosis of *Staphylococcus aureus* independently of complement activation (Neth *et al.*, 2002). However, it is unclear whether MBL is acting as a direct opsonin for microorganisms (Kuhlman *et al.*, 1989; Polotsky *et al.*, 1997) or is enhancing other pathways such as complement - (CR1) or immunoglobulin - (Ig) receptor mediated phagocytosis (Tenner *et al.*, 1995; Ghiran *et al.*, 2000). Indeed, it is still unclear whether MBL receptors are distinct from receptors for the structurally similar C1q molecule (Bajtay *et al.*, 2000).

1.5.2 MBL and inflammation

MBL has also been proposed to display anti-inflammatory properties by modulating the cytokine production by monocytes. Addition of low levels of MBL to whole blood resulted in increases of TNF- α , IL-1 β and IL-6 production, whereas higher doses of MBL suppressed the production of all three inflammatory cytokines (Jack *et al.*, 2001, Turner 2003). It has also been found that MBL binds to apoptotic cells and stimulates their ingestion by phagocytes (Ogden *et al.*, 2001).

1.5.3 The common opsonic defect and MBL deficiency

The common opsonic defect and eventually MBL deficiency was first reported in 1968 when Miller *et al.* described the case of a child, who had suffered from recurrent upper respiratory tract infections, diarrhoea and failure to thrive in the first two years of life

(Jacobs and Miller, 1972). The patient did not appear to have a deficiency of immunoglobulin but, in the presence of the patient's serum neutrophils from human donors were unable to phagocytose *Saccharomyces cerevisiae* (Baker's yeast) efficiently. The patient's neutrophils were able to phagocytose efficiently when the serum of other donors was used in the same assay. There was a familial association of the opsonic defect, although the other family members were healthy. The patient was treated with plasma infusions, which ameliorated the condition. Further studies showed the abnormality was present in approximately 5-8% of the general population (Soothill and Harvey 1976; Levinsky *et al.*, 1978). The frequency of this defect was higher in recurrent unexplained infections, but the defect was also present in healthy adults with no history of persistent infection.

In the early 1980's, it was known that the complement system is activated by two or more immunoglobulin G molecules or one IgM molecule bound to a target surface (classical pathway) or by the binding of hydrolysed C3 to a permissive surface such as a bacterium (alternative pathway). Activation in this manner would lead to opsonisation of the target by the amplified deposition of multiple opsonic C3 fragments or the direct lysis of Gram negative bacteria by formation of macromolecular complex, MAC (Walport *et al.*, 2001). Since a gross immunoglobulin defect did not appear to be cause of the opsonic defect, and in any case would be unlikely in such a large proportion of the population, a study of complement deposition was begun. Biochemical assays using the D-mannose polymer zymosan confirmed that the absence of a serum factor led to the poor deposition of C3b and iC3b on the yeast surface (Turner *et al.*, 1981) without

deficiency of any known component of either the alternative or classical pathways of complement activation (Turner *et al.*, 1991).

MBL appeared to be an ideal a candidate molecule for the serum factor deficient in patients with the common opsonic defect. It was demonstrated these individuals were deficient in MBL and that the addition of purified MBL to the serum of these patients resulted in increased deposition of C3b, Factor B and C4 on zymosan (Super *et al.*, 1989).

When mannan, a predominant polysaccharide in zymosan, was used in complement binding assays to study a population of blood donors, defective opsonisation was associated with low levels of MBL and the role of the novel lectin pathway of complement activation was established (Super *et al.*, 1989; 1990). The importance of a newly recognised complement defect was detailed by Turner and co-workers (Turner *et al.*, 1991).

1.6 THE ROLE OF MBL IN INFECTIOUS DISEASE

1.6.1 Disease model of MBL deficiency- MBL mouse knock-out models

To evaluate a causal role of MBL in host susceptibility to infection mouse models of MBL deficiency were created. Although human MBL deficiency was first report in 1989, the existence of two functional genes in rodents- *mb1-1* and *mb1-2* that encode for MBL-A and MBL-C, respectively delayed the generation of MBL-deficient animals.

These two forms of rodent MBL bear 50 % homology to one another and are both able to activate MBL-MASP pathway (Liu *et al.*, 2001). The generation of double knock-out (DKO) MBL deficient mice was recently reported (Shi *et al.*, 2004, Takahashi *et al.*, 2006). The authors found 100% of MBL-null mice died 48 hours after exposure to an intravenous inoculation of *Staphylococcus aureus* compared with 55% mortality in wild-type mice. Importantly, pre-treatment of MBL-null mice with recombinant human MBL (rhMBL) restored MBL-MASP pathway *in vivo*. This study provides evidence that the presence of low-producing *MBL-2* gene haplotypes might be a genetic factor that predisposes certain humans to poor outcomes as a result of *S. aureus* infection.

To study the effects of MBL deficiency during viral infection a model of generalized *herpes simplex virus -2 (HSV-2)* infection was chosen (Gadjeva *et al.*, 2004a). MBL-A and MBL-C DKO mice was infected with *HSV-2* via the intraperitoneal route. DKO mice cleared *HSV-2* from the liver less effectively than the comparable wild-type animals. Reconstitution with rhMBL before and during the *HSV-2* infection dramatically reduced the viral titres in the liver. The data showed that MBL modulates the response to *HSV-2* in mice by affecting neutralization of the virus. This suggests that lack of MBL-mediated complement activation increases susceptibility to viral infection.

These results provide *in vivo* evidence that MBL plays role in first line host defense against infection with *S. aureus* and *HSV-2* and support the clinical associations between MBL deficiency and increased susceptibility to certain infections.

1.6.2 The human studies-the role of MBL in health and disease

MBL deficiency has been genetically defined as possession of haplotype associated with decreased levels, which applies to ~ 40% of the population (Madsen *et al.*, 1994; Madsen *et al.*, 1995). Profound MBL deficiency (<400 ng/ml) occurs in ~7-10 % of the Caucasian population (Turner, 1998). MBL deficiency is thought to be one of the most common immunodeficiencies. The fact that most MBL deficient people do not get infectious has led to speculation that a second immune defect needs to be present to render the individual susceptible to infection. In support of an earlier suggestion, Aittoniemi and co-workers found that MBL deficiency only presented clinically when there was an associated immunodeficiency such as IgG subclass deficit (Aittoniemi *et al.*, 1998). There is evidence that severe infections were more common in patients with combined deficiencies, either IgA or any of the IgG subclasses or IgA and MBL deficiency (Santaella *et al.*, 2005).

The impact of MBL deficiency on the wellbeing of the general population has been questioned recently. In 2003, Tacx *et al.* investigated 177 unselected adults hospitalised with fever. They found microbial infection induced complement activation independently of MBL and concluded that MBL deficiency does not predispose to serious infection in unselected adults (Tacx *et al.*, 2003). The impact of variant MBL haplotypes among the general population was also questioned in the recent study involving 9245 Danish adults. The authors found no significant difference in morbidity and mortality in MBL deficient individuals versus controls (Dahl *et al.*, 2004). Studies by Ross *et al.* offered one potential explanation for these observations. They found that

antibody-mediated activation of the classical pathway of complement compensated for impaired target opsonization *via* the MBL pathway in MBL deficient individuals (Ross *et al.*, 2004). These studies further confirm that MBL deficiency may only become clinically relevant when it occurs in combination with defects in other arms of immune system.

The high frequency of MBL deficiency in the general population suggests that defective MBL mediated immunity can be compensated by alternative defence strategies. This is not true of deficiencies of some immune components. For examples, patients with X-linked agammaglobulinaemia suffer from severe and repeated bacterial infections, particularly those caused by encapsulated organisms. It would appear that MBL deficiency may not be as important as some other immune components, because alternative means of eradicating pathogens have evolved. For example there are many ways to kill bacteria. Immunoglobulin, independently and in association with the complement system, can effectively destroy bacteria in the absence of MBL. While MBL may enhance opsonisation, is not absolutely required.

The role of MBL in disease was first appreciated within paediatric population and the most rigorous MBL clinical research has been conducted in this group. Low serum MBL and *MBL-2* mutations have been implicated in childhood infection. This association has been demonstrated in children with mild respiratory tract infections managed within community (Koch *et al.*, 2001), in more severe infections requiring hospital admission (Summerfield *et al.*, 1997), as well as in the paediatric intensive care unit setting where

MBL deficiency was associated with a greater risk of developing the Systemic Inflammatory Response Syndrome (SIRS), (Fidler *et al.*, 2004).

The time during which MBL is most relevant for protection against infection is probably infancy and early childhood. The hospital based study found a significant association throughout childhood (0 to 18 years of age), (Summerfield *et al.*, 1997), whereas in the community based-study the influence of MBL deficiency was strongest in children between 6 and 17 months of age (Koch *et al.*, 2001). This later study showed a greater risk of infection in MBL deficient infants, at a time when passively acquired maternal immunity has waned but adaptive antibody responses are still immature (time of transient hypogammaglobulinaemia of infancy). This has led to the concept of a “window of vulnerability”, whereby the importance of MBL in host defence is greatest when adaptive immune responses are deficient.

Another area of uncertainty concerns the precise level of MBL that confers protection, and below which children are at risk. Early studies using the functional opsonisation assay identified 5-10 % of the population as deficient and therefore at risk; this corresponds to a protein level of <100 ng/ml (Soothill and Harvey 1976; Levinsky *et al.*, 1978). Garred *et al.* (1995) found only homozygotes for structural alleles to be at risk; the corresponding protein concentration was < 20 ng/ml (Garred *et al.*, 1995). Another study, however, has been reported that heterozygotes to be at risk as well (Summerfield *et al.*, 1997). It has been shown that MBL levels in the heterozygote group range from nil to 1.2 µg/ml, thus providing a range of protection in individuals.

1.7 MBL and SUSCEPTIBILITY TO INFECTION

The role of MBL in increased susceptibility to various bacterial, viral and fungal infections has been studied in both paediatric and adult populations (Summerfield *et al.*, 1997; Turner *et al.*, 2000; Kilpatrick *et al.*, 2002; Eisen *et al.*, 2003). MBL deficiency has been implicated in both disease susceptibility and as a modulator of severity:

- Chronic hepatitis B (Thomas *et al.*, 1996; Yuen *et al.*, 1999),
- Chronic hepatitis C (Matsushita *et al.*, 1998),
- HIV infection (Garred *et al.*, 1997),
- *Plasmodium falciparum* (Luty *et al.*, 1998),
- *Meningococcal* infection (Hibberd *et al.*, 1999),
- Cystic fibrosis (Garred *et al.*, 1999; Gabolde *et al.*, 2001; Davies *et al.*, 2004),
- *Cryptosporidium parvum* (Kelly *et al.*, 2000),
- Visceral Leishmaniasis (Santos *et al.*, 2001),
- Systemic Inflammatory Response Syndrome (SIRS) in adults (Garred *et al.*, 2003)
- SIRS in children (Fidler *et al.*, 2004),
- *HSV-2* (Gadjeva *et al.*, 2004).

Interestingly, MBL deficiency has also been suggested to have a protective effect against certain diseases in populations where variant alleles are frequently observed (Santos *et al.*, 2001).

An association between MBL deficiency, poor lung function and shortened life span in cystic fibrosis (CF) patients was reported, and presumed to be connected with severity of pulmonary infections *P. aeruginosa*, *B.cepacia* (Garred *et al.*, 1999; Gabolde *et al.*, 2001). Thus, MBL has been identified as a modifier of severity in cystic fibrosis (CF), although studies have produced differing results and the mechanism of action remains (Aittoniemi *et al.*, 1996) unclear. A recent study (Davies *et al.*, 2004) involving large cohort of adults and children has explored this relationship further. Adults with two structural mutations, but not heterozygotes, had significantly reduced lung function and oxygen saturations, more frequent hospital admissions and raised systemic inflammatory markers. This observation was not related to increased rates of infection with *P. aeruginosa*, and there was no increased susceptibility to *B.cepacia*. Interestingly, none of these findings were mirrored in the paediatric cohort. Severe MBL deficiency appears to be detrimental to CF adults, although heterozygotes are not affected. Different results were obtained from paediatric cohorts. The group of homozygotes was very small (n=6) and the study was probably underpowered to detect a difference. Garred *et al.*, (1999) observed an MBL effect in individuals as young as 8 years old, in contrast most of the children in the Davies *et al.* study had well-preserved lung function, and low or undetectable levels of systemic inflammatory markers (C-reactive protein (CRP), white cell counts).

MBL variant alleles were associated with an increased risk of developing sepsis, severe sepsis, septic shock and death in adults (Garred *et al.*, 2003). Further, Fidler and co-workers reported that MBL variant alleles were associated with an increased risk of developing Systemic Inflammatory Response Syndrome (SIRS) and of progression to sepsis and septic shock in children (Fidler *et al.*, 2004).

1.7.1 Changes in serum MBL levels with age

Terai et al, 1993 studied a total of 1085 normal Japanese sera from individuals ranging in age from 3 to 100 years old were measured for MBL levels (Terai *et al.*, 1993). A high age dependency in serum MBL levels was observed. MBL levels declined from age group 3-9 to 20-29 years old, but remained constant after 30-39 years old. Thus, MBL levels of the younger groups (less than 20 years old) were apparently higher than those of the older (more than 20). Aittoniemi and colleagues investigated the changes occurring in the serum of MBL in infancy, during later childhood and in adults. The serum concentration of MBL increased significantly after birth, and was at its highest (the mean 8.13 mg/L) at the age of 1 month. After that, it declined to the initial level until the age of 5 months. The MBL levels continued to decrease during childhood, and after the age of 12 years the MBL values reached the adult level (Aittoniemi *et al.*, 1996). In a study by Kilpatrick *et al.*, (1996) measured MBL concentration in 237 umbilical cord blood samples and compared to adults controls consisted of sera prepared from consecutive blood donations (n=352), (Kilpatrick *et al.*, 1996). MBL levels in cord blood samples were found to be significantly lower than those of blood donors. Therefore, it should be stressed that age has to be taken into consideration in the evaluation of MBL levels.

1.7.2 Changes in serum MBL levels in early life

Determination of MBL levels in children (less than 3 years old), including neonates, have been carried out (Terai *et al.*, 1993; Thiel *et al.*, 1995; Lau *et al.*, 1995; Frakking *et al.*, 2006), in order to determine how and when the MBL levels change during early life.

Terai and co-workers have studied perinatal changes in serum MBL levels, from day 0 to day 5 in term neonates (Terai *et al.*, 1993). A considerable amount of MBL is present in serum at birth and it has been observed an increase in MBL levels after birth during the first 5 days of life. The serum MBL in term neonates reaches its highest level within the first week after birth (2.48 µg/ml) when levels are almost equal to that observed in an older (3-9 year) study group (Terai *et al.*, 1993). In a study by Thiel and co-workers MBL levels were measured in term neonates through the first 12 months of life (Thiel *et al.*, 1995). It has been reported that levels of MBL increase during the first 3 months of life to achieve levels comparable to that observed in adults (Thiel *et al.*, 1995). On average, the level of MBL at term was 37% of the post 3-month level. They also showed that in premature neonates, MBL levels increased 3-fold from the time of birth until the 10th week of life. According to Aittoniemi *et al.*, MBL concentration increased significantly after birth with highest levels detected at 1st month of age (Aittoniemi *et al.*, 1996). Then, the MBL concentration declined to the initial level until the age of 5 months.

To date, only a few studies have investigated MBL levels as a function of gestational age in preterm neonates (Malhorta *et al.*, 1994; Lau *et al.*, 1995; Hilgendorff *et al.*, 2005; Frakking *et al.*, 2006). Malhotra and co-workers measured MBL levels in amniotic fluid samples obtained vaginally in patients with ruptured membranes following the spontaneous onset of labour (Malhotra *et al.*, 1994). Patients' gestational age ranged from 26 to 41 weeks of gestation. Interestingly, Malhotra *et al.*, found a sharp increase in MBL levels in the amniotic fluid at approximately 32 weeks of gestation, suggesting MBL may play a role in the antibody-independent recognition and clearance of

pathogens in the amniotic cavity, thus providing protection to the foetus in the amniotic cavity from ascending vaginal infection, particularly towards term.

A large study to determine the developmental profile of MBL in preterm neonates was performed by Lau and co-workers (Lau *et al.*, 1995). MBL was measured in 885 longitudinally collected serum samples from 168 preterm neonates, and 63 were genotyped for the codon 54 mutation. They postulated that there is a maturation process in MBL levels for those without the codon 54 mutation from preterm to term neonates and to adults. However, for those with the codon 54 mutation, there was a significant difference in MBL levels between preterm and term neonates, but not between term neonates and adults. This observation suggests that the maturation process for MBL levels for those preterm infants with the codon 54 mutation is probably completed by full-term gestation. The two recently published studies confirmed that preterm neonates are MBL deficient at birth compared to term neonates and MBL levels increase with gestational age (Hilgendorff *et al.*, 2005; Frakking *et al.*, 2006). Summary of studies on MBL in early life are summarized in Table 4.2 (Chapter 4).

1.7.3 MBL and infection in early childhood

There are two hypotheses regarding the role of MBL in natural immunity and infection. The so-called “ante-antibody” concept proposed by Ezekowitz envisages a role immediately following primary contact with any organism during the days before the first antibody appears (Ezekowitz *et al.*, 1988). The alternative hypothesis argues for a major role during the “window of vulnerability” experienced by all infants following the

decline in levels passively acquired maternal antibodies and preceding the maturation of the infant's own antibody repertoire (Super *et al.*, 1989; Turner *et al.*, 1991).

Kilpatrick *et al.*, (1998) found that MBL may be important for innate immunity and in some cases of sudden infant death syndrome (SIDS) where bacterial infection may have contributed to SIDS. Therefore, relative MBL deficiency might be associated with susceptibility to SIDS. The proportion of infants with low MBL values was similar in SIDS infants and controls. However, the mean MBL levels for the SIDS group were higher than that of the controls. This difference could be interpreted as an acute phase response (Kilpatrick *et al.*, 1998).

Kielgast *et al.*, 2003 investigated whether MBL in umbilical cord blood (UCB) is associated with incidence rate of hospitalizations for infections during early childhood. It was a follow-up study from birth to 31 months of age. A total of 2104 infants were enrolled. Rate of hospitalization with infections in children with low levels of MBL was similar to hospitalization because of other diseases. Low levels of MBL in UCB were associated with hospitalization in general and particularly with hospitalization for viral infections (Kielgast *et al.*, 2003). Other hospital based-studies carried by Koch *et al.* (2001) have found that increased susceptibility to acute respiratory tract infections (ARIs) in children below 2 years of age is associated with low levels of MBL. A 2.08-fold increased relative risk of ARI was largely restricted to children aged 6 to 17 months while the effect amongst 0-5 month age was minimal. This suggests that genetic factors such as MBL deficiency may play an important role particularly during the vulnerable period of childhood when the adaptive immune system is immature.

1.8 AIMS OF THIS THESIS

A major aim of this thesis is to investigate the role and contribution of MBL to the risk of bacterial infections in early life (preterm neonates) and in Human Immunodeficiency Virus (HIV) in children.

The specific aims of this study are:

- 1) To explore the influence of MBL gene polymorphisms on the susceptibility and severity of infection in preterm neonates
- 2) To evaluate the developmental profile in MBL levels during the 1st month of life as a function of gestational age and birth weight
- 3) To investigate a role of MBL in the susceptibility and progression of HIV disease.

CHAPTER 2

METHODOLOGY

2.1 MBL GENOTYPING

2.1.1 DNA extraction

Genomic DNA was extracted from 200 µl whole blood using a commercial kit (QIAamp DNA blood Mini Kit, Qiagen, Crawley, UK) according to the manufactures instructions. The QIAamp DNA purification procedure comprises three steps and is carried out using QIAamp spin columns in a standard microcentrifuge (M-24 BOECO, Germany). Briefly, 200 µl whole blood was mixed with 20 µl of Qiagen Protease in a 1.5 ml microcentrifuge tube. 200 µl Buffer AL was added to the sample and mixed by pulse-vortexing for 15 s and heated at 56 ° C in a water-bath (Grant Instruments, Cambridge, UK) for 10 minutes. 200 µl of absolute ethanol (Sigma-Aldrich, Poole, UK) was added and the resulting solution was vortexed and centrifuged (6 500 rpm, 1 min). The supernatant obtained after centrifugation was applied to a sterile spin column (held in a collection tube) and re-centrifuged (6500 rpm, 1 min). Specific binding of genomic DNA to the spin column allowed contaminants to be discarded in the collection tube. The spin columns were placed in clean 2 ml collection tubes (provided in kit). 500 µl of Wash buffer AW1 was added to the column and re-centrifuged (6 500 rpm, 1 min) followed by a second wash with 500 µl of wash buffer AW2 and re-centrifuged (13 000 rpm; 3 min.). The spin columns were removed and placed in clean 1.5 ml tubes before elution of genomic DNA from the column by the addition of 200 µl of sterile water. Extracted DNA (approx.10 ng/µl) was stored at -20 °C until further use.

2.1.2 MBL genotyping by heteroduplex generation

Detection of mutations by molecular techniques allows one to correlate genotype with phenotypic changes in an organism leading to a better understanding of mode of disease inheritance. In mutation detection, test DNA molecules are compared for differences from reference wild-type DNA. In this study, PCR of specific regions followed by hetero-duplexing was used to identify specific MBL mutations. Previous methods for genotyping have included restriction enzyme analysis of polymerase chain reaction (PCR) products (Lipscombe *et al.*, 1992; Madsen *et al.*, 1994), probing with radiolabelled allele specific oligonucleotides (Madsen *et al.*, 1994), and the amplification refractory mutation system (Davies *et al.*, 1995).

Intra-strand base pairing causes single-stranded DNA molecules to form complex secondary structures. Alteration of the nucleotide sequence of the molecule by as little as a single base can reshape the secondary structure, with consequent changes in electrophoretic mobility. Hetroduplexing exploits the differences in mobility between wild-type and mutant strands of DNA. The sequence of interest is amplified by PCR from genomic DNA. The PCR DNA product is denatured and then analyzed by agarose gel electrophoresis under non-denaturing conditions at neutral pH. A wild-type molecule generates two bands corresponding to each single strand of the PCR product.

2.1.2.3 Heteroduplex generators

Cross-matching an unknown DNA with a specially constructed third party DNA, termed a universal heteroduplexes generator (UHG) was adapted from Wood *et al.*, (1993). These are synthetic DNA sequences that mimic a genomic DNA sequence, but which contain nucleotide substitutions, deletions or insertions at nucleotide positions opposite to and contiguous (upstream and downstream) with known mutation sites within genomic DNA. The UHG and genomic DNA sequences are amplified with the same PCR primes, and are hybridised together in equal proportion post-PCR by heating and slow cooling. This generates DNA heteroduplexes having different conformational forms, and thus different electrophoretic mobilities, characteristic for individual alleles of a gene (Wood *et al.*, 1993).

In the present study MBL exon-1 genotyping was done by specific amplification of exon-1 by polymerase chain reaction (PCR). In a separate reaction a synthetic DNA (UHG) based on exon-1 was also amplified using the same primer set. The two reaction products were combined, denatured and annealed to form homo and heteroduplexes before being subjected to polyacrylamide gel electrophoresis. The data from heteroduplexing procedures were combined to give haplotypes comprising one of the three structural mutations (O) or wild-type (A) together with the X/Y promoter polymorphism.

2.1.3 PCR amplification of genomic DNA and UHG

The exon-1 mutation spanning and the X/Y promoter region were amplified by PCR using genomic DNA as template. The conditions for the PCR reactions have previously been optimized (Jack *et al.*, 1997; Turner *et al.*, 2000). Also, similar PCR reactions were performed for the UHG. Routinely, the PCR reaction was performed in 20 µl total volume using; 5 µl of aqueous DNA (approximately 10 ng/ µl), 0.6 µl each of 50 µM MBL primers (Genosys Biotechnologies Ltd., Pampisford, Cambs, UK):

Sense- MBL - (5'- CCAACACGTACCTGGTTCC-3'),

Antisense -MBL - (5'- CTGTGACCTGTGAGGATGC-3'),

2 µl of 10 X PCR buffer, 2 µl of dNTPs mix (2 mM each of dATP, dCTP, dGTP and dTTP) (Invitrogen, Paisley, Scotland), 1.2 µl of 25 mM MgCl₂, 0.2 µl of AmpliTaq Gold DNA polymerase (Roche Molecular Systems, Branchburg, New Jersey, USA) and sterile water (Sigma, UK).

The PCR reactions for the promoter polymorphisms were conducted under similar conditions using the following primers (Genosys Biotechnologies Ltd., Pampisford, Cambs, UK):

Sense MBL promoter - (5'- AGGCATAAGCCAGCTGGCAAT-3 '),

Antisense MBL promoter - (5'- CTAAGGAGGGGTTTCATCTG-3')

PCR reactions were carried out in a thermacycler (Phoenix, Helena BioSciences, Sunderland, UK) and the conditions were 95 ° C for 15 minutes, followed by 35 cycles of 95 ° C for 45 seconds, 56 ° C for 45 seconds and 72 ° C for 45 seconds. These were followed by a final extension step at 72 ° C for 10 minutes.

2.1.3.1 PCR amplification of UHG

Using the commercial UHG as the template, supplied by MWG-Biotech AG (Ebersberg, Germany) large scale PCR reactions were initially performed to obtain quantities of stock UHG generator that could be utilized in a series of subsequent reactions. Reagents used for large scale prep were: ~ 5 µg of the appropriate UHG template,

18 µl of 50 µM MBL primers:

Sense MBL - (5' - CCAACACGTACCTGGTTCC-3'),

Antisense MBL - (5' - CTGTGACCTGTGAGGATGC-3')

A separate reaction for the promoter UHG region was performed:

18 µl of 50 µM MBL primers:

Sense MBL promoter - (5' - AGGCATAAGCCAGCTGGCAAT-3'),

Antisense MBL promoter - (5' - CTAAGGAGGGGTTCATCTG-3')

100 µl of 10 X PCR buffer, 100 µl of dNTPs mix, 60 µl of 25 mM MgCl₂, 2.6 µl of AmpliTaq Gold DNA polymerase, and 785 µl sterile water.

PCR reactions were carried out at 95 °C for 15 minutes, followed by 35 cycles of 95 °C for 45 seconds, 56 °C for 45 seconds and 72 °C for 45 seconds. These were followed by a final extension step at 72 °C for 10 minutes.

2.1.4 Analysis of PCR products by agarose gel electrophoresis

All PCR products were routinely tested for integrity on 2 % agarose gels prior to heteroduplexing and genotyping. Agarose gels were prepared as follows: 2 g Agarose (Sigma- Aldrich) was dissolved in 100 ml Tris- Borate- EDTA Buffer (TBE), (stock solution, 10 X concentrate, Sigma- Aldrich) by microwave heating. Agarose was cooled

before addition of 10 µg ethidium bromide (stock solution=5 mg/ml, Sigma, Poole, UK) and set in appropriate casting tray. 1 µl of 6 X Loading Buffer (Promega, Southampton, UK) was added to 3 µl of PCR product. A 1 kilobase Ladder was included as a marker (Promega, Southampton, UK). The gels were run in TBE buffer at 100 V for 1 hour. PCR products were visualised using the AlphaImager system (Alpha Innotech, San Leandro, CA, USA).

2.1.5 MBL heteroduplexing by polyacrylamide gel electrophoresis

Following confirmation of specific amplification of genomic and UHG DNA, 10 µl of UHG PCR product plus 5 µl of loading dye were added to 15 µl of each DNA sample. The combined samples were heated to 95 ° C for 10 minutes and then allowed to cool to room temperature over 20 minutes to allow slow and specific annealing. 15 µl of the heteroduplex product was loaded onto a 20 % polyacrylamide gel for genotyping. The gel was prepared as follows:

20 ml 30 % Acrylamide/ Bis Acrylamide, stock solution (Severn Biotech LTD, UK), 6 ml of 10 x TBE Buffer (Sigma, Poole, UK), 300 µl 10 % ammonium persulfate solution (National Diagnostic, UK), 30 µl TEMED (N, N, N, N, -tetramethylethylenediamine), (Sigma, Poole, UK) and 5 ml MiliQ water. The gel was allowed to set (acrylamide polymerisation for 5-10 min. at room temperature). After polymerization was complete, the gels were attached to the electrophoresis tank, using clips. The notched plate faced inward toward the buffer reservoir. Heteroduplex samples were loaded and run for 16 hours at 140 V in TBE buffer at room temperature. Gels were stained using 0.5 µg/ ml

ethidium bromide in TBE buffer for 10 minutes before photography under UV light (Alpha Imager system, Alpha Innotech, San Leandro, USA).

As well as faster homoduplex formation, this technique also allows heteroduplex formation between two different DNA molecules. Due to the known mutations in the promoter and exon-1 region of the MBL gene one may expect two different heteroduplexes per allele with differing electrophoretic mobilities. Each allele should generate two different DNA heteroduplexes corresponding to the two DNA strands from each allele. Homozygotes generated two heteroduplex bands and heterozygotes generated four heteroduplex bands which were visualized and quantified. A representative polyacrylamide gel showing heteroduplexing pattern for X/Y promoter polymorphism obtained in the present study is shown below (Figure 2.1).

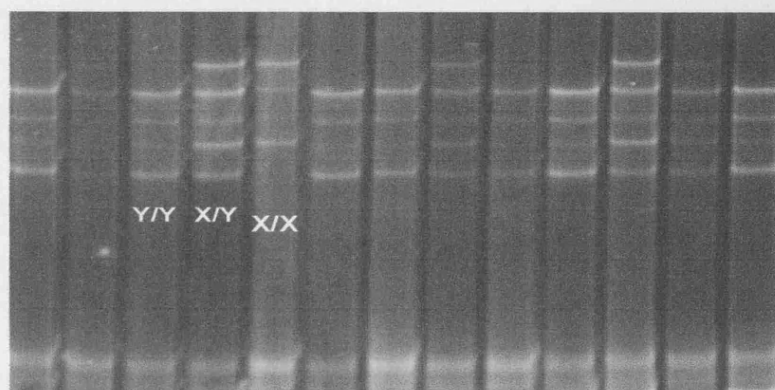


Figure 2.1 Polyacrylamide gel showing promoter polymorphism Y/Y, X/ Y, X/ X (performed by Researcher A.D.)

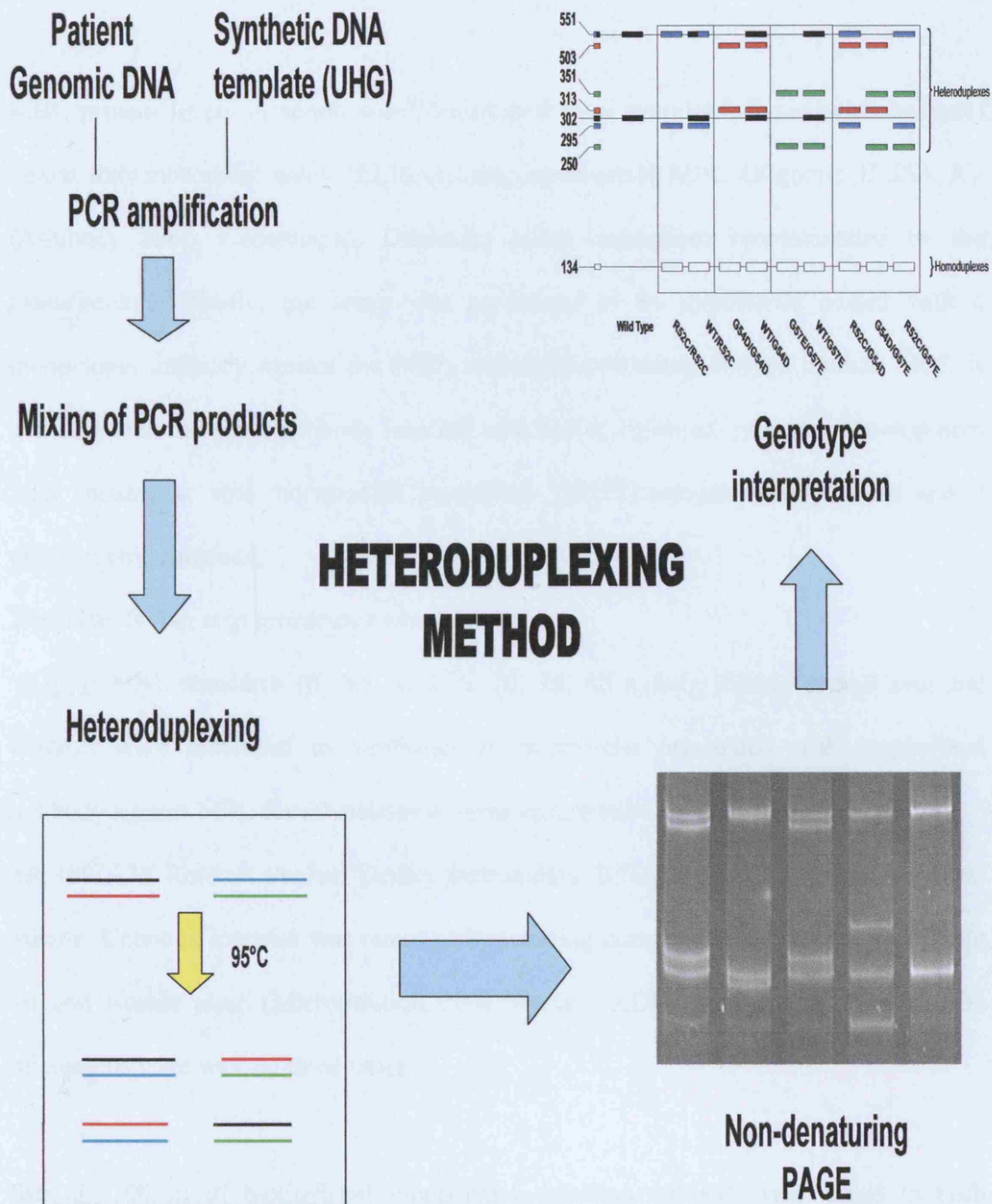


Figure 2.2 Diagram presenting summarized methods of MBL genotyping

2.2 MBL PHENOTYPE

MBL protein levels in serum were determined by a symmetrical sandwich enzyme-linked immunosorbent assay (ELISA) using commercial MBL Oligomer ELISA Kit (Antibody Shop, Copenhagen, Denmark) using instructions recommended by the manufactures. Briefly, the assay was performed in 96 microwells coated with a monoclonal antibody against the MBL carbohydrate-binding domain. Bound MBL is detected with the same antibody labelled with biotin, followed by colour development after incubation with horseradish peroxidase (HRP)-conjugated streptavidin and a chromogenic substrate.

The assay is four step procedures which are:

Step 1: MBL standards (0, 0.5, 1, 2, 5, 10, 20, 40 ng/ml), diluted patient sera and controls were incubated in duplicates in microwells pre-coated with monoclonal antibody against MBL for 60 minutes at room temperature (RT) on a shaking platform (R 100/ TW Rotatest Shaker, Denley Instruments, Billingham, UK) set at 200 Revs/minute. Unbound material was removed by washing using wash solution provided with kit and washer plate (Microtitration Plate Washer, ADIL Instruments, France). The microwells were washed three times.

Step 2: 100 µl of biotinylated monoclonal detection antibody was added to each microwell and incubated for 60 minutes at RT on a shaking platform. Unbound antibody was removed by washing using wash solution (as described in step 1).

Step3: 100 µl of HRP-conjugated streptavidin was added to each test well and incubated for 60 minutes at RT on a shaking platform (200/ minute). This step allowed complex formation with the bound biotinylated antibody. Unbound conjugate was removed by washing using wash solution (as described above)

Step 4: 100 µl of chromogenic peroxidase substrate containing tetramethylbenzidine (TMB) was added to each well and incubated for exactly 15 minutes at RT in the dark. The bound HRP- streptavidin reacts with the substrate to generate a colored product. The enzymatic reaction was stopped chemically by adding 100µl Stop solution (provided). The color intensity was read at 450 nm in an ELISA reader (MRX, Dynatex Laboratories,) within 30 minutes. The optical density readings obtained for the standard MBL were utilised to construct a calibration curve from which the concentrations of MBL in the test specimens were calculated.

2.3 STATISTICAL ANALYSES

Data were analyzed using SPSS version 12.0 (SPSS, Inc, Chicago, IL, USA). Patients were classified according to their MBL exon 1 mutations as wild-type (A/A), heterozygous (A/O), or homozygous (O/O). Genotype (or allele) frequencies were analysed by the Chi-square test. Graphs and summary measures were used to look at distribution of data. The methods used in descriptive statistics include histograms and normal plots. As analyzed data were found non-normally distributed, summary were presented as median and interquartile range (IQR). Non-parametric tests: Mann-Whitney and Kruskal-Wallis were used in analysis. Differences between groups (MBL serum levels) were compared with the Mann-Whitney U Test (for two groups) or Kruskal-Wallis test (three or more groups). Relations between different variables were tested using the Spearman rank correlation test. Probability values were two-sided, and significance was defined as $p < 0.05$. MBL levels are presented in the text and graphically as median values. Graphs demonstrate median (bar), interquartile range (IQR); (box) and range (whiskers). Data points that lay more than 1.5 IQRs outside the IQR were marked individually as outliers. Statistical analyses presented in Chapter 4 were performed with SPSS version 12.0 (SPSS, Inc, Chicago, IL, USA) and with SAS version 9.1 (SAS®, Cary, NC, USA). The analyses of the risk of sepsis according to MBL levels, birth weight and gestational age at birth were performed using logistic regression.

CHAPTER 3

MBL IN HIV INFECTION IN CHILDREN: RELATION TO DISEASE PROGRESSION AND HIGHLY ACTIVE ANTIRETROVIRAL THERAPY (HAART)

3.1 INTRODUCTION

Human immunodeficiency virus (HIV) infection still represents one of the most important health problems in the world. The spread of HIV infection in developed countries is a consequence of sexual lifestyle, intravenous drug use, and treatment with contaminated blood products. According to the World Health Organization ('WHO') estimates and the Joint United Nations Programme on HIV/ AIDS ('UNAIDS') report *2007 AIDS Epidemic Update*:

- In 2007, some 33.2 million individuals were living with HIV worldwide, compared with 39.4 million in 2004;
- 2.5 million individuals had become newly infected and 2.1 million individuals had died of AIDS (compared with 3.1 million in 2004);
- 2.5 million children were estimated to be living with HIV in 2007 (unchanged from 2004), 90 % of them in Africa.

These data reflect advances in methodology that have resulted in substantial revisions from previous estimates. The epidemic estimates presented in the report reflect improvements in country data collection and analysis, as well as a better understanding of the natural history and distribution of HIV infection. In the past few years a number of countries, most notably in sub-Saharan Africa and Asia, have expanded and improved their HIV surveillance systems, conducting new, more accurate studies that provide more precise information about HIV prevalence than earlier studies. In addition, 30 countries mostly in Africa have conducted national representative population-based household surveys. These have also informed adjustments for other countries with

similar epidemics that have not conducted these surveys. New assumptions have also been made as a result of a better understanding of the natural history of untreated HIV infection.

The single biggest reason for the reduction in global HIV prevalence figures in the past year was the recent revision of estimates in India after an intensive reassessment of the epidemic in that country. The revised estimates for India combined with important revisions of estimates in five sub-Saharan African countries (Angola, Kenya, Mozambique, Nigeria, and Zimbabwe) account for 70% of the reduction in HIV prevalence as compared to 2006 estimates.

In recent years Highly Active Antiretroviral Therapy (HAART) has dramatically improved clinical, virologic and immunological parameters in HIV-infected individuals, although a favourable response is not achieved in all patients (Tozser, 2001). Importantly, HAART does not eradicate the virus, and for several immunologic parameters, there is no normalization during therapy (Blankson, 2002). Thus, additional treatment modalities are needed and immunomodulatory therapy in combination with HAART may represent such an option. Several studies have begun to explore a potential immunological role for MBL in HIV infection. These studies are discussed in more detail below.

3.1.1 Direct MBL and HIV-1 viral interactions

The role of MBL in HIV disease has been extensively studied both *in vitro* and *vivo*, but has resulted in somewhat conflicting findings. It was first demonstrated *in vitro* that HIV infection of CD4 + T lymphocytes was impaired by physiological concentrations of MBL (Ezekowitz *et al.*, 1989). In 1989, Ezekowitz and colleagues observed binding of purified MBL to HIV infected cell lines and MBL inhibited HIV infection of lymphoblasts (Ezekowitz *et al.*, 1989).

Several studies have investigated the molecular interactions between MBL and HIV. HIV virus envelope proteins, such as HIV gp120 and gp41, contain carbohydrates that enable binding to MBL. HIV gp 120 is highly glycosylated with about half of the 120kDa molecular weight consisting of N-linked glycans. In contrast, there are only four or five potential N-linked carbohydrate sites on gp41. MBL is now known to recognize gp120 in a sugar specific manner *via* its' CRD since the interaction could be inhibited by EDTA, mannan or N-glycanase treatment (Ezekowitz *et al.*, 1989; Saifuddin *et al.*, 2000; Thielens *et al.*, 2002; Hart *et al.*, 2003).

MBL binds to both CCR5-tropic and CXCR4 tropic primary isolates (PI) of whole virus (Saifuddin *et al.*, 2000). Binding of virus to MBL required expression of gp120/gp41 on virus particles, suggesting the presence of either a usually high carbohydrate density and/or a unique carbohydrate structure on gp120/gp41 that is a target of MBL. The study by Saifuddin and co-workers suggested that MBL can selectively interact with HIV *via*

the high-mannose glycosylation sites of gp120/gp41 which play a major role in MBL-HIV interactions (Saifuddin *et al.*, 2000).

These results indicated that MBL recognizes high-mannose glycans known to be present on gp 120 in the domain that is recognized by CD4 cells and thereby inhibits viral entry to susceptible cells. *In vitro*, binding of gp120 to the cell-surface marker, CD4, on the surface of the T cells is inhibited by MBL. This neutralizing effect is related to complement, but is independent of C1q, or antibodies or viral lysis (Haurum *et al.*, 1993). Association of MBL with the viral surface may lead to activation of the lectin pathway and viral lysis (Haurum *et al.*, 1993).

The recent study, by Ying and co-workers has reported the MBL binds to both PI and cell line-adapted HIV but does not effect neutralization by levels of MBL normally present in serum. However, binding and opsonization of HIV by MBL may alter virus trafficking and viral-antigen presentation during HIV infection (Ying *et al.*, 2004). MBL may influence uptake by DCs which express a cell surface lectin called “dendritic cell-specific intracellular adhesion molecule 3 (ICAM-3)-grabbing non-integrin” (DC-SIGN). DC-SIGN has been shown to mediate a type of infection called “trans” infection, when DCs bind HIV and efficiently transfer the virus to T cells (Geijtenbeek *et al.*, 2000; Geijtenbeek and van Kooyk, 2003). A recent study showed that preincubation of HIV strains with MBL, at levels similar to those found in normal human serum, could inhibit the HIV infection of T cells mediated by DC-SIGN (Spear *et al.*, 2003).

3.1.2 MBL and susceptibility to HIV infection in adults

It has been suggested that MBL deficiency, as assessed by MBL serum concentrations, increases susceptibility to HIV infection in adults (Nielsen *et al.*, 1995; Garred *et al.*, 1997; Prohaszka *et al.*, 1997). Nielsen *et al.*, studied a population of 80 HIV infected patients and 123 normal healthy individuals. The number of HIV positive individuals with undetectable MBL levels was significantly higher than in healthy controls (Nielsen *et al.*, 1995). Two studies conducted by Garred and co-workers in European and African population respectively reported that MBL deficiency, as assessed by genetic methods, was more frequent in HIV infected individuals when compared to healthy controls (Garred *et al.*, 1997a, b). HIV infected patients were more likely to be homozygous for MBL variant alleles (Garred *et al.*, 1997a). Another study performed by Pastinen *et al.* 1998 in a Finish population suggested also that homozygosity for the *MBL* variant alleles occurred more frequently in HIV infected patients (Pastinen *et al.*, 1998). A recent study performed by Vallinoto and co-workers in a Brazilian population has provided further evidence that homozygosity for the *MBL* variant alleles is associated with HIV-1 transmission (Vallinoto *et al.*, 2006). Several other studies, however, did not find similar associations (Senaldi *et al.*, 1995; Maas *et al.*, 1998; Malik *et al.*, 2003). For example, Senaldi *et al.*, 1995 found that MBL levels were higher in HIV infected individuals than normal controls. In another study by Malik *et al.*, 2003, MBL genotype frequencies were similar in HIV-infected and control individuals in a Colombian population. Table 3.1 shows published adults studies related to HIV disease (Tables 3.1).

3.1.3 MBL and HIV disease progression in adults

The effect of MBL on HIV disease progression has also been investigated (Senaldi *et al.*, 1995; Nielsen *et al.*, 1995; McBride *et al.*, 1998). A number of studies have failed to show any effect of MBL status on disease progression (Senaldi *et al.*, 1995; Nielsen *et al.*, 1995; McBride *et al.*, 1998). However, a few studies actually reported a weak protective effect of *MBL-2* mutant alleles on progression to AIDS and death (Maas *et al.*, 1998; Prohaszka *et al.*, 1998). Mass and co-workers showed that HIV infected patients with the *mbi* variant alleles progressed more slowly to AIDS (Maas *et al.*, 1998). Prohaszka reported that MBL levels negatively correlated with CD4+ T lymphocytes (percentage and absolute number) suggesting that MBL deficiency could be beneficial (Prohaszka *et al.*, 1998). Interestingly, Garred and colleagues found that individuals with one or more *MBL* variant alleles had a shorter survival time after a diagnosis of AIDS in an adult Danish homosexual population (Garred *et al.*, 1997a). A recent study by Vallinoto and co-workers has also showed that normal MBL serum concentrations were required for control of viraemia and therefore may act to limit a disease progression (Vallinoto *et al.*, 2006).

A few studies have attempted to examine MBL status in HIV-infected patients in the context of Antiretroviral Therapy (ART) (Hundt, *et al.*, 2000; Heggelund *et al.*, 2003). In a study by Hundt *et al.*, 2000, normal MBL levels were found in six long term nonprogressors (LTNPs). They concluded that possession of wild type MBL genotype is protective against HIV infection (Hundt, *et al.*, 2000). Only one study to date has been performed in the era of treatment with HAART (Heggelund *et al.*, 2003). The authors

observed an increase in MBL levels in advanced HIV disease suggesting that MBL may modulate HIV disease progression. Table 3.1 shows published adults studies related to HIV disease (Table 3.1).

3.1.4 MBL and HIV infection in paediatric studies

A clear difference in natural HIV progression between adults and children has been observed with respect to transmission, pathogenesis and clinical manifestations (Scott *et al.*, 1984). Studies investigating the role of MBL in paediatric HIV infection are limited. Amaroso and colleagues showed that the codon 54 polymorphism in the *MBL-2* gene was associated with AIDS progression (Amaroso *et al.*, 1999). Another study by Boniotto and co-workers found *MBL-2* promoter polymorphisms influenced both the risk of infection as well as disease progression (Boniotto *et al.*, 2000).

An association between the presence of any one of the three exon-1 *MBL-2* alleles and HIV infection in perinatally exposed Brazilian children has also been reported (Boniotto *et al.*, 2003). A study by Lian *et al.*, 2004 showed that MBL concentrations were increased in more severe clinical stages of the HIV disease (Lian *et al.*, 2004). Table 3.2 shows published paediatric studies related to HIV disease (Table 3.2).

| Study | Patient population (n) | Assay | Findings | |
|--------------------------------|------------------------|-------------------|---|---|
| | | | Susceptibility | Severity |
| Senaldi <i>et al.</i> , 1995 | 92 Europe | ELISA | MBL levels higher in HIV infected patients | No difference in MBL levels between CDC groups. No correlation with CD4+T cells. |
| Nielsen <i>et al.</i> , 1995 | 80 Denmark | ELISA | Undetectable MBL levels in HIV infected patients | No association |
| Garred <i>et al.</i> , 1997 a) | 173 Africa | ELISA | Low MBL levels associated with increased risk of HIV infection | Not done |
| Garred <i>et al.</i> , 1997 b) | 96 Denmark | Genotype ELISA | Higher rate of homozygous carriers of <i>mbi</i> variant alleles | Homozygous- <i>mbi</i> variant alleles had shorter survival time after AIDS diagnosis |
| Prohaszka <i>et al.</i> , 1997 | 67 Europe | ELISA | Low MBL levels in HIV infected patients | Negative correlation between MBL levels and CD4+ T cells |
| Maas <i>et al.</i> , 1998 | 131 Netherlands | Genotype | Not done | Homozygous- <i>mbi</i> variant alleles progressed more slowly to AIDS |
| McBride <i>et al.</i> , 1998 | 131 UK | Genotype ELISA | No association | No association |
| Pastinen <i>et al.</i> , 1998 | 300 Finland | Genotype | Increased rate of homozygous - <i>mbi</i> variant alleles in HIV (+) ve | Not done |
| Malik <i>et al.</i> , 2003 | 278 Colombia | Genotype | No association | Not done |
| Heggelund <i>et al.</i> , 2003 | 62 Norway | ELISA | Not done | MBL levels markedly elevated in CDC group C |
| Vallinoto <i>et al.</i> , 2006 | 145 Brazil | Genotype | Association of variant B with HIV infection | Lower CD4+ T cells & higher viral load in carriers of <i>mbi</i> B allele. |

Table 3.1 Studies investigating MBL and HIV infection in adults

Table 3.2 Summary of studies relating MBL status and HIV infection in children

| Study | Patient population (n) | Assay | Findings | |
|-------------------------------|--|---------------------------------|--|--|
| | | | Susceptibility | Severity |
| Amaroso <i>et al.</i> , 1999 | 52 HIV (+) ve & 27 exposed HIV(-) ve Italy | Genotype codon 54 | Higher frequency of variant alleles in HIV infected patients | Presence of variant alleles associated with rapid progression |
| Boniotto <i>et al.</i> , 2000 | n=90; vertically acquired HIV infection Italy | Genotype Promoter-Position -550 | Promoter polymorphism associated with vertical transmission of HIV | Homozygous-variant alleles associated with rapid progression to AIDS |
| Boniotto <i>et al.</i> , 2003 | n=114 perinatally HIV infected & 27 exposed HIV(-) ve Brazil | Genotype - exon1 | Variant <i>mbi</i> gene alleles more frequent in HIV infected patients | Not done |
| Lian <i>et al.</i> , 2004 | n=127 Brazil | ELISA | Not done | Trend for higher MBL levels in more severe patients (CDC stage C) |

3.1.5 Long-Term Non-Progressors (LTNP) in HIV infection

Initial studies of HIV-infected persons focused mainly on individuals presenting with symptoms of AIDS, who therefore represent the most severely affected end of the spectrum disease. From adults' studies, over time it has been possible to identify individuals who are infected with HIV and yet whose immune system remains undamaged > 20 years following infection. In these Long-Term non-Progressors (LTNP), HIV replication is suppressed to very low levels due to effective immune responses (Cytotoxic T lymphocytes, T-helper cells or neutralising antibody responses) and/or the host genetic factors that play an important role in susceptibility to HIV-1 infection and in the rate of disease progression i.e. CCR5 and CXCR4 genes (HIV-1 co-receptors); (Alvarez *et al.*, 1998). A balance between the host factors and virus factors are major determinants of the outcome of HIV infection.

Paroli and co-workers defined LTNP HIV-infected individuals characterized by the absence of disease with low viral loads and stable or even increasing CD4 (+) T cell counts for prolonged periods of time. In these subjects, an HIV-specific immune response which is either stronger or directed against a wider array of viral epitopes than that seen in progressors, can be observed (Paroli *et al.*, 2001).

According to definition by Suzuki and Yamamoto, 1997 the length of time between HIV infection and development of AIDS was considered to be 10 years on average, but a few percent of infected persons-so-called LTNP, did not develop AIDS even more than 10 years after infection. In LTNP it has been described that 1) there is a low viral load, 2)

isolated HIV is relatively non virulent type, 3) antibody to HIV do not enhance virus propagation, 4) type 1 cytokines is more dominant than type 2, and 5) antiviral activity of CD8+ T cells is strong. In LTNP the immune-responses actively suppress HIV proliferation, so that viruses are controlled at low level (Suzuki and Yamamoto, 1997).

Rodes and co-workers described the main features of LTNP and presented longitudinal virology and immunology follow-up for a 6-years period. Cohort of 19 patients with evidence of non- progressive HIV-1 infection was established in 1997 in reference HIV/AIDS center in Madrid. All patients had serologically proven HIV-1 infection for at least 10 years, repeated CD4 cell counts $> 500 \times 10^6$ cells/l, and no prior history of HIV-related symptoms, in the absence of any antiretroviral therapy. All of these individuals have been prospectively followed every 4–6 months since January 1997 (Rodes *et al.*, 2004).

HIV-1-positive LTNP who were enrolled in the Australian LTNP study, had been infected with HIV-1 for at least 8 years and maintained a CD4+ T cell count of $> 500 \times 10^6$ /l in the absence of anti-retroviral therapy. HIV-1 progressors were selected from the Sydney AIDS prospective study and consisted of individuals who were infected at around the same time and known to have progressed to AIDS and/or death within 8 years of HIV-1 infection (Clegg *et al.*, 2000).

Paediatric studies on LTNP are more complicated. Earlier studies have shown the speed of progression to disease in paediatric HIV infection is related to extent of progression in the mother at the time of birth (Blanche *et al.*, 1997). More than 90 % of HIV-1

infection in children was acquired by mother-to-child-transmission (Scarlati, 1996). Approximately 20 % of HIV-1 infected children born to seropositive mothers' progressed rapidly during the first year of life towards development of AIDS (progression to U.S. Centers for Disease Control and Prevention (CDC) group C disease or HIV- related death), and 4.7% per year thereafter, giving a cumulative incidence of 36% by 6 years (Blanche *et al.*, 1997).

Studies in North America and Europe, prior to implementation of antiretroviral therapy (ART), identified a group ~25% of children infected around the time of birth who progress very rapidly to AIDS within one year of life (European Collaborative Study); (Sharland *et al.*, 1997). Of the remaining 75%, the median survival time was 7 years (Barnhart *et al.*, 1996). In sub-Saharan Africa, the figures indicated that at least 89% of children infected with HIV perinatally died with AIDS by the age of 3 years (Taha *et al.*, 2000). Observation of cohorts of perinatally infected children showed that there are children who have survived well beyond the median life expectancy of 8 years for those in developed countries (pre-therapy) who have had not developed AIDS in the first year of life. However most of these children have high levels of virus and it was not possible to study mechanisms which control viremia.

According to Vigano and co-workers, 1997 some children who are infected with HIV-1 during the perinatal period remain asymptomatic for very long periods in the absence of antiretroviral treatment, as is the case for some adults. Vertically infected children showing resistance to disease progression are immunologically and virologically distinct from those in whom progressive HIV infection is observed. LTNP were defined in vertically infected children $> \text{ or } = 8$ years of age without disease progression or mild

symptoms and an absolute CD4⁺ T cells count \geq 500/microliter with CD4⁺ percentage \geq 25% (Vigano *et al.*, 1997). LTNPs maintain a very low viral load in plasma, virus is less cytopathic and they have predominant T-helper cell profile and their cellular CD8 T cell antiviral response is strong (Romiti *et al.*, 2000).

The recently published study by Warszawski *et al.*, 2007 reported the proportion of children who developed neither symptoms nor major immunological perturbations to the age of \geq 10 years in a prospective French Pediatric Cohort of 568 HIV-1 infected children who had been observed since birth. Children with long-term non-progression of infection (LTNPs) were defined as HIV-1 infected children who had been observed for at least 10 years, never received antiretroviral treatment other than zidovudine monotherapy, never developed symptoms of Centers for Disease Control and Prevention clinical category C or B, and had a CD4⁺ cell percentage of $<$ 25% no more than once during follow-up (Warszawski *et al.*, 2007).

3.2 AIMS

The aims of the study described in this chapter were:

- 1) To access the MBL genotype distribution in the UK cohort of HIV-infected children
- 2) To explore the potential correlation between the course/ severity of HIV infection and MBL genotype and phenotype
- 3) To investigate the role of MBL in the progression of HIV disease in HIV Long Term non-Progressors (LTNPs).

3.3 PATIENTS and METHODS

3.3.1 Patients

One-hundred and twenty-eight HIV infected children (64 females) attending the HIV Family Clinic at Great Ormond Street Hospital for Children NHS Trust, (GOSH) London, UK between January 2003 and August 2004 were recruited. Approval for the study was granted by the Great Ormond Street Hospital for Children/ Institute of Child Health Research Ethics Committee and parental written consents were also obtained. At the time of blood sampling, the patients' clinical notes were assessed and all children included were classified according to the Centres for Disease Control and Prevention (CDC; Atlanta, GA, USA) revised classification (clinical and immunological) system detailed in Tables 3.3 and 3.4 for HIV infection in children (CDC, Revised Classification, *MMWR* 1994).

Children who had not taken ART were defined as antiretroviral therapy naive (ARTN). To identify HIV infected children in our studied cohort at GOSH who are LTNP the definition of paediatric LTNP was adopted from ongoing parallel LTNP study coordinated by Professor Philip Goulder from John Radcliffe Hospital, Oxford. This was multi-centre, including patients from GOSH, longitudinal study of LTNP investigating protective HIV specific immune responses. The aim of the multi-centre LTNP study was to identify and study perinatally infected children who represent the best <5% in terms of non-progression to disease, relative preservation of the immune system despite HIV infection, and successful control of viremia.

Children who are antiretroviral therapy naive (ARTN) were defined for the purpose of this study as LTNP who fulfill the following criteria:

- ≥ 24 months of age and
- Viral load $< 10,000$ copies of HIV-1 RNA/ml at enrollment on the LTNP study and
- CD4% $\geq 20\%$ at enrollment on the LTNP study.

Table 3.3 Classification of HIV infected children according to clinical symptoms
(1994 Revised, MMWR)

Table 3.4 Classification of HIV infected children according to immunological impairment (1994 Revised, MMWR)

3.3.2 Samples collection

Whole blood was collected in lithium heparin tubes for serum isolation. The isolation procedure involved centrifugation of whole blood (M-24 BOECO, Germany; 2 000 rpm, 7 min). The supernatants were aliquoted into fresh tubes and stored at - 80 ° C. Whole blood was also collected in an EDTA tubes and stored at -80 ° C until required for DNA extraction. DNA was isolated and frozen at -20 ° C until required for MBL genotyping. The immunological and virological markers of disease progression (CD4+T cells, CD8+T cells, CD3+T cells, HIV-1 RNA viral load) and MBL measurements (genotype and phenotype) were performed for each patient.

3.3.3 Methods

The methods used for assessing MBL genotype and phenotype and statistical analyses are described in detail in Chapter 2. Chi-square or Fisher's exact test was used to compare proportions in two groups (MBL genotype in HIV studied group and published control groups). The analysis was performed using Stata version 10 statistical programme (StataCorp, USA).

3.4 RESULTS

3.4.1 Patient characteristics

One-hundred and twenty-eight HIV infected children (64 females) were enrolled. MBL genotyping was performed in all 128 children and serum MBL levels were measured in 127. The median age at enrolment was 8.9 years (range 1.4-16.1 years). Seventy-nine percent of children were of Sub-Saharan origin, 7 % were white, 9 % were of mixed origin (white and African, or white and unknown) and 5 % for whom place of birth or either biological parent were unknown. Table 3.5 shows patients' characteristics according to the CDC clinical and immunological classification (Table 3.5).

Eighty-three individuals were receiving or had received HAART. Of the 45 individuals who were classified as ART naive (ARTN), 10 were LTNPs. The relation between patients ART status and CDC classification is shown in Table 3.6. Children who were not classified as ARTN or LTNPs had taken HAART, and therefore described as the HAART group.

Table 3.5 Classification of HIV-1 infected children based on clinical and immunological parameters.

| Clinical category, n (%) | | | | Immunological category, n (%) | | |
|--------------------------|-----------|-----------|-----------|-------------------------------|------------|----------|
| | A | B | C | 1 | 2 | 3 |
| Male | 19 (48.7) | 30 (51.7) | 15 (48.4) | 38 (49.3) | 22 (47.8) | 4 (80.0) |
| Female | 20 (51.3) | 28 (48.3) | 16 (51.6) | 39 (50.7) | 24 (52.2) | 1 (20.0) |
| Total | 39 | 58 | 31 | 77 | 46 | 5 |

Table 3.6 Classification of HIV-1 infected children based on therapy, clinical and immunological parameters.

| Clinical category, n (%) | | | | Immunological category, n (%) | | |
|--------------------------|-----------|-----------|----------|-------------------------------|-----------|----------|
| | A | B | C | 1 | 2 | 3 |
| LTNPs | 5 (12.8) | 5 (8.6) | 0 | 7 (9.1) | 3 (6.5) | 0 |
| ARTN | 19 (48.7) | 16 (27.6) | 0 | 19 (24.7) | 15 (32.6) | 1 (20.0) |
| HAART | 15 (38.5) | 37 (63.8) | 31 (100) | 51 (66.2) | 28 (60.9) | 4 (80.0) |
| Total | 39 | 58 | 31 | 77 | 46 | 5 |

3.4.2 HIV and MBL genotype

All 128 individuals were genotyped for *MBL-2* mutations. Table 3.7 shows the genotype frequencies of structural gene variants in the *MBL-2* in this patient cohort. 77.3 % of the patients were homozygous for wild type MBL alleles. The 22.7% of the patients were heterozygous (18%) and 4.7 % homozygous for variant MBL alleles. The C allele was predominant. It was found in 76 % of heterozygous (A/O) individuals compared to patients with the B (9.6%) and D (14.4 %) alleles (Table 3.7). To explore the difference in gene distribution between different ethnic groups, the genotype analysis was performed for the whole study population and separately for Black African population (the majority of the children in the study, n=101).

Table 3.7 MBL exon-1 genotype frequencies in HIV-1 infected patients

| MBL exon-1 genotype | Studied group Total n= 128 (%) | Studied African n=101 (%) |
|----------------------------|---------------------------------------|----------------------------------|
| A/ A | 99 (77.3) | 74 (73.3) |
| A/ B | 2 (1.6) | 2 (2.0) |
| A/ C | 17 (13.3) | 16 (15.8) |
| A/ D | 4 (3.1) | 3 (3.0) |
| A/ O | 23 (18.0) | 21 (20.8) |
| B/ B | 0 | 0 |
| C/ C | 4 (3.1) | 4 (4.0) |
| D/ D | 0 | 0 |
| B/ C | 0 | 0 |
| C/ D | 2 (1.6) | 2 (2.0) |
| O/ O | 6 (4.7) | 6 (6.0) |

Additionally, we analysed the genotype frequencies including the promoter polymorphism at position -221 in the *MBL-2* gene in this patient cohort (Table 3.8). The -221 promoter polymorphism (X/Y) was chosen as it greatly influences protein level (Madsen *et al.*, 1995). No significant additional effect of the X or Y variant promoter on susceptibility to HIV infection was observed. Structural and promoter alleles in studied cohort and other populations are shown in Tables 3.15 and 3.16.

Table 3.8 MBL-2 structural and promoter alleles in the studied group

| <i>MBL-2</i> genotype | All (n=128) n (%) | African (n=101) n (%) |
|----------------------------------|------------------------------|----------------------------------|
| YA/ YA | 64 (50) | 47 (46.5) |
| YA/ XA | 33 (25.8) | 26 (25.7) |
| XA/ XA | 2 (1.6) | 1 (1.0) |
| YA/ Y0 | 18 (14.1) | 17 (16.8) |
| XA/ Y0 | 5 (3.9) | 4 (4.0) |
| Y0/ Y0 | 6 (4.7) | 6 (5.9) |

3.4.3 MBL and disease status

The relationship between *MBL-2* genotype and disease status was examined. The immunological and viral markers in all patients were analysed and documented at the time of recruitment. There was no significant difference in HIV viral load or immune phenotype between patients with a wild type genotype or with one or more variant *MBL-2* alleles (Table 3.9).

Table 3.9 Correlation between *MBL-2* genotypes, immunological and viral characteristics in HIV-1 infected children

| Immunological markers (x 10⁶/ L)* | <i>MBL-2</i> wild- type alleles (n= 99) | <i>MBL-2</i> variant alleles (n= 29) | <i>P</i>-values |
|---|---|--|------------------------|
| CD3 T cells | 1.970 (1360-2440) | 1660 (1265-2315) | 0.24 |
| CD4 T cells | 670 (400-960) | 540 (350-690) | 0.33 |
| CD8 T cells | 1090 (820-1430) | 1030 (620-1435) | 0.56 |
| Viral markers | | | |
| HIV-1 RNA copies x 10 ³ /L | 3900 (50-24 900) | 1300 (50-19 541) | 0.52 |

* Values in median (IQR), Mann-Whitney Test. P values < 0.05 indicate significance.

Similarly, when the *MBL-2* genotype was analysed with respect to the CDC clinical classification no obvious differences were observed (chi-square test; p=0.14); (Table 3.10).

Table 3.10 Distribution of *MBL-2* genotypes in CDC paediatric clinical categories

| <i>MBL-2</i> genotypes | Patients, n (%) | CDC A n (%) | CDC B n (%) | CDC C n (%) |
|-------------------------------|------------------------|--------------------|--------------------|--------------------|
| YA/ YA | 64 (50.0) | 23 (59.0) | 23 (39.7) | 18 (58.1) |
| YA/ XA | 33 (25.8) | 8 (20.5) | 18 (31.0) | 7 (22.6) |
| XA/ XA | 2 (1.6) | 0 | 2 (3.4) | 0 |
| YA/ Y0 | 18 (14.1) | 7 (17.9) | 7 (12.1) | 4 (12.9) |
| XA/ Y0 | 5 (3.9) | 1(2.6) | 2 (3.4) | 2 (6.5) |
| Y0/ Y0 | 6 (4.7) | 0 | 6 (10.3) | 0 |
| Total | 128 (100) | 39 (100) | 58(100) | 31(100) |

However, when patients were classified according to their age-specific CDC CD4+ T cells immunological categories, patients in categories 2 and 3 (< 25% CD4+ T cells) were more likely to have *MBL-2* variant alleles (chi-square test; $p=0.01$; Table 3.11).

Table 3.11 Distribution of *MBL-2* genotypes in CDC Immunological categories

| <i>MBL-2</i> genotypes | Patients, n (%) | CD4 category 1 (> 25 %), n (%) | CD4 category 2 (15- 24 %), n (%) | CD4 category 3 (< 15 %), n (%) |
|-----------------------------------|----------------------------|---|---|---|
| YA/ YA | 64 (50) | 45 (58.4) | 18 (39) | 1 (20) |
| YA/ XA | 33 (25.8) | 17 (22.1) | 14 (30.4) | 2 (40) |
| XA/ XA | 2 (1.6) | 1 (1.3) | 0 | 1 (20) |
| YA/ Y0 | 18 (14.1) | 11(14.3) | 7(15.2) | 0 |
| XA/ Y0 | 5 (3.9) | 1 (1.3) | 4 (8.7) | 0 |
| Y0/ Y0 | 6 (4.7) | 2 (2.6) | 3 (6.5) | 1(20) |
| Total | 128 (100) | 77 (100) | 46 (100) | 5 (100) |

From these results it would appear that MBL deficiency is more frequent in patients with severe disease as assessed by CD4+ T-cells status. To examine these further, patients who were classified as LTNPs were analysed.

3.4.4 MBL and Long Term Non-Progression in HIV infected children

HIV infection doesn't inevitably lead to the development of AIDS (Levy *et al.*, 1993).

While the majority of HIV infected individuals progress to AIDS, a minority of patients do not show signs of disease and can maintain a stable CD4+ T cells count for at least 10 years of infection. These patients are known as LTNPs (Vigano *et al.*, 1997).

The studied HIV population was divided into three groups: LTNP-s (n=10), ARTN (n=35) and HAART (n=83). The characteristics of the patients in therapy groups are shown in Table 3.12.

Table 3.12 Detailed characteristics of the HIV-1 infected patients in therapy groups

| | LTNP-s | ARTN | HAART | P-values |
|---|--------------------|-------------------|-------------------|-----------------|
| n (%) | 10 (7.81) | 35 (27.34) | 83 (64.84) | 0.32 |
| Males/females | 6/ 4 | 15/ 20 | 43/ 40 | 0.54 |
| Age (years) | 9.72 (8.25-13.12) | 7.79 (5.76-10.97) | 9.10 (5.64-12.74) | 0.21 |
| CD4 T cells | 545 (337-657) | 500 (320-830) | 690 (412-980) | 0.22 |
| CD8 T cells | 1070 (885-1407) | 1160 (930-1620) | 1060 (730-1350) | 0.36 |
| HIV-RNA copies x 10³/ L | 4.98 (0.049-21.12) | 21.38 (8.6-40.4) | 0.08 (0.049-6.08) | 0.0001 |

Values in median (IQR), Kruskal-Wallis test. *P*-values <0.05 indicate significance.

LTNP-s were found to have CD4+ and CD8+ T cell counts in the same range as that of subjects who were ARTN and HAART. The HIV-RNA viral load in LTNP-s was significantly lower than that observed for the ARTN group ($p < 0.05$).

Tables 3.13 and 3.14 represent MBL genotype and phenotype analyses in this HIV cohort.

Table 3.13 *MBL-2* structural and promoter alleles in therapy groups

| <i>MBL-2</i> genotype | n (%) | LTNPs, n (%) | ARTN, n (%) | HAART, n (%) |
|----------------------------------|--------------|-------------------------|------------------------|-------------------------|
| YA/ YA | 64 (50) | 2 (20) | 21 (60) | 41 (49.4) |
| YA/ XA | 33 (25.8) | 7 (70) | 5 (14.3) | 21 (25.3) |
| XA/ XA | 2 (1.6) | 0 | 1 (2.9) | 1 (1.2) |
| YA/ Y0 | 18 (14.1) | 1 (10) | 5 (14.3) | 12 (14.5) |
| XA/ Y0 | 5 (3.9) | 0 | 1 (2.9) | 4 (4.8) |
| Y0/ Y0 | 6 (4.7) | 0 | 2 (5.7) | 4 (4.8) |
| Total | 128 (100) | 10 (100) | 35 (100) | 83 (100) |

In children on HAART, the prevalence of *MBL-2* gene mutations was higher than in other groups; HAART vs. LTNP (p= 0.3); HAART vs. ART (p= 0.2); ART vs. LTNP (p=0.8).

Of the ten patients classified as LTNP, only, one showed an *MBL-2* mutation (A/D). Interestingly, this patient had an MBL level of 611ng/ ml. Overall, the proportion of *MBL-2* genotypes was not significantly different between the groups: LTNP-s, ARTN and HAART (Chi- square test; p= 0.17).

Table 3.14 Concentrations of serum MBL in the therapy groups (p=0.57,

Kruskal Wallis test)

| MBL serum levels (ng/ ml) | LTNP (n= 10) | ARTN (n =35) | HAART (n= 83) |
|---------------------------------------|-------------------------|-------------------------|---------------------------|
| Median (ng/ ml) | 2 991 | 2 005 | 2 416 |
| IQR | 1517- 3926 | 771- 3929 | 1101- 4937 |

Normal range of MBL serum concentrations were maintained in the LTNPs (IQR, 1517-3926 ng/ ml) group. In comparison, MBL serum concentration assigned as low ($MBL \leq 300$ ng/ ml), intermediate (MBL between 300 and 1300 ng/ ml), and normal ($MBL > 1300$ ng/ml) was observed in 9 (10.8%), 13 (15.7%), and 61 (73.5%) of 83 HIV-1 infected children who were receiving HAART. This proportion of MBL phenotypes was consistent with MBL genotype analysis.

In conclusion, 10 patients were classified as being LTNP. Only one of these patients had an *MBL-2* gene mutation, suggesting that wild type MBL may contribute in maintaining the disease status in this group of patients. Normal MBL serum concentration in the LTNPs adds further support to our hypothesis.

3.4.5 HIV disease and MBL levels

The relationship between MBL levels and genotype was explored. Serum MBL levels were significantly related to *MBL-2* genotype ($p < 0.001$). Fig 3.1 shows the correlation of serum MBL levels to *MBL-2* genotype in all studied patients (Figure 3.1). To further investigate the role of MBL in HIV disease status MBL levels were analysed in relation to the CDC clinical and immunological categories. No significant differences in MBL levels were found between the groups when classified according to their CDC clinical status ($p=0.45$). Similarly, no significant difference in MBL levels was found between the groups when classified according to their CD 4+ T cells immunological categories ($p=0.12$; Figure 3.2). However, interestingly higher levels of MBL were observed in patients within the CD4+ T cells category 3 ($CD4+ T cells < 15\%$) group when compared with category 1 and 2 (Figure 3.2).

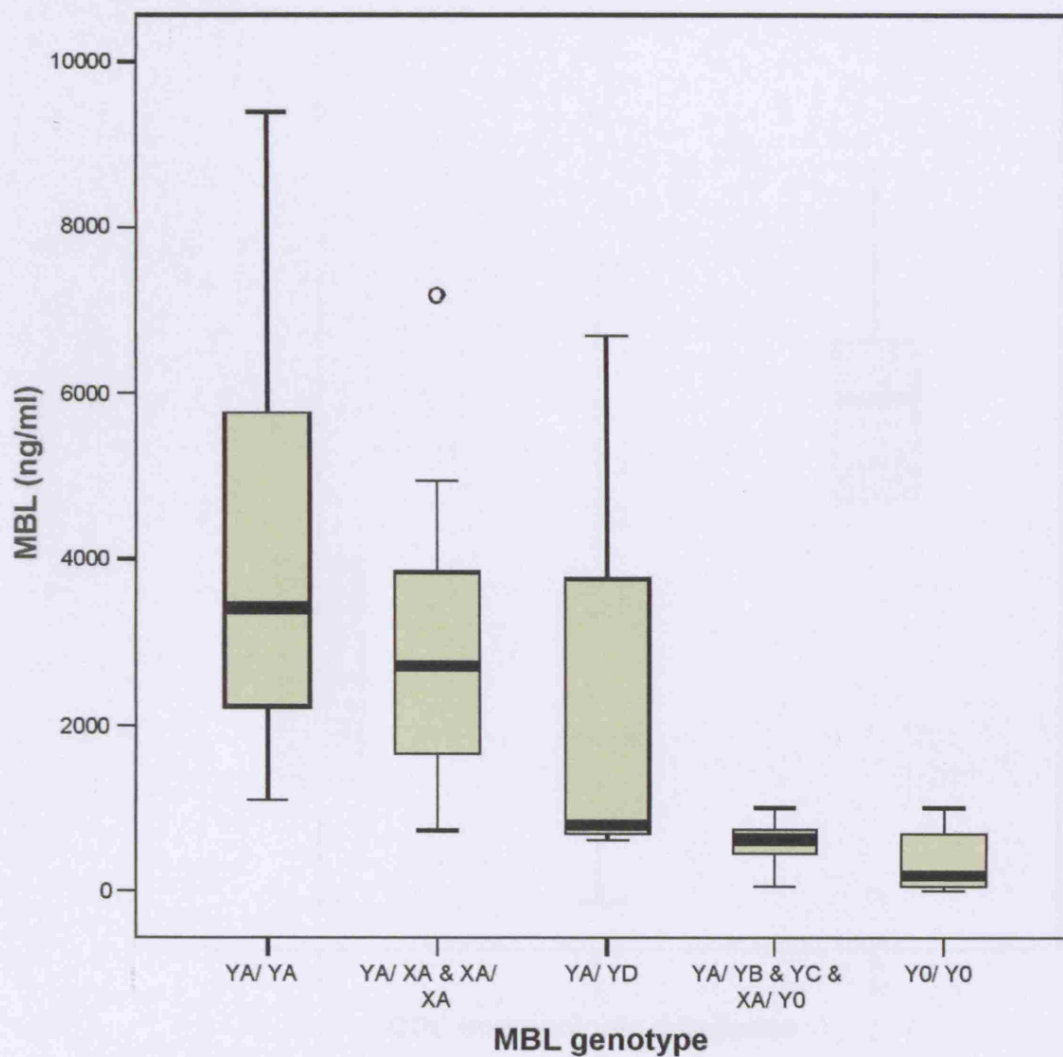


Figure 3.1 Correlation of serum MBL levels to *MBL-2* genotype in paediatric HIV infection ($p < 0.001$). Box-whisker plot of MBL serum concentrations (ng/ml) in HIV-infected children. The boxed area represents 50% of values. The thick line inside the box represents the median value. Whiskers are 10-th and 90-th percentiles. Outliers are open circles.

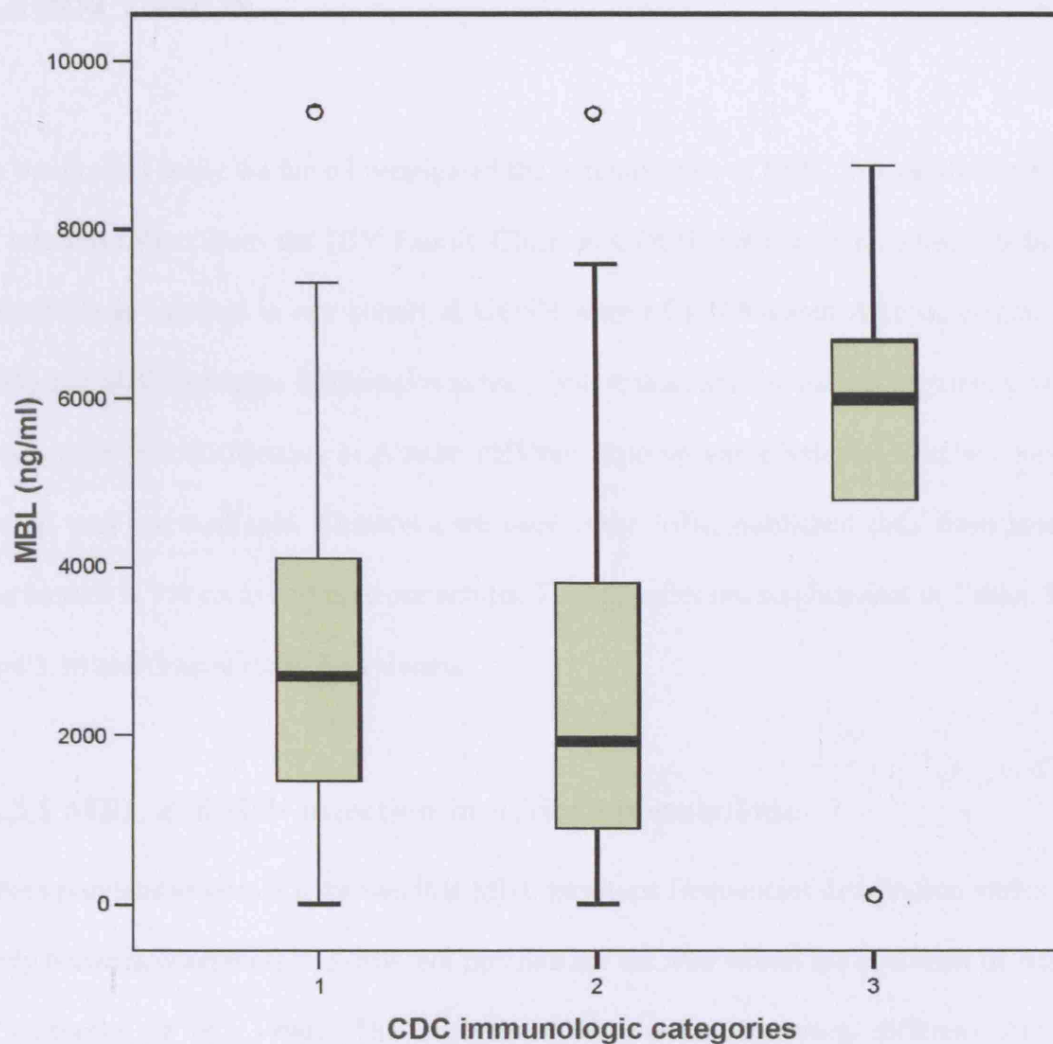


Figure 3.2 Serum MBL levels in patients classified according to CDC immunological categories of HIV infection. CDC Category 1, n= 76; Category 2, n= 46; Category 3, n= 5; (Kruskal-Wallis; P= 0.117). Box-whisker plot of MBL serum concentrations (ng/ml) in HIV-infected children. The boxed area represents 50% of values. The thick line inside the box represents the median value. Whiskers are 10-th and 90-th percentiles. Outliers are open circles.

3.5 DISCUSSION

In the present study we have investigated the potential role of MBL in a paediatric HIV-1 infected cohort from the HIV Family Clinic at GOSH, London, UK. Most (79 %) of the children enrolled in our cohort at GOSH were of sub-Saharan African origin. We analysed MBL genotype frequencies in the whole cohort and we looked separately at the MBL genotype distribution in African children. Appropriate ethnically matched control group was not available. Therefore we used other MBL published data from studies performed in Africa to compare our results. These studies are summarized in Tables 3.15 and 3.16 and discussed in more details.

3.5.1 MBL and HIV infection in African populations

From previous studies it is known that MBL genotype frequencies distribution varies not only between continents and different populations but also within the continent of Africa (Lipscombe *et al.*, 1996). The analysis of frequencies between different African populations by geographical location (Tables 3.15 and 3.16) showed highly significant differences in MBL gene distribution, both wild type (WT) and gene mutation (A/ O, O/O) between Gabon population and Ghanaians from West Africa, ($p < 0.0001$). Similarly, there was significant difference in MBL gene distribution between Soweto population, South Africa and Ghanaians, West Africa, ($p < 0.0001$).

Table 3.15 MBL exon-1 genotype frequencies in HIV-1 infected patients and other populations

| MBL gen | Studied group Total n= 128 (%) | Studied African n=101 (%) | Gabonese HIV-uninfected n= 120 (%) | Gabon Total n=188 (%) | Soweto South Africa n=168 (%) | Ghanaians West Africa n= 551 (%) | Oxford controls n= 353 (%) |
|----------------|---------------------------------------|----------------------------------|---|------------------------------|--------------------------------------|---|-----------------------------------|
| A/ A | 99 (77.3) | 74 (73.3) | 80 (66.7) | 124 (66.0) | 109(64.9) | 257 (46.6) | 213 (60.3) |
| A/ B | 2 (1.6) | 2 (2.0) | 4 (3.3) | 4 (2.1) | 4 (2.3) | 3 (0.5) | 73 (20.7) |
| A/ C | 17 (13.3) | 16 (15.8) | 34 (28.3) | 53 (28.2) | 50 (29.8) | 234 (42.5) | 7 (2.0) |
| A/ D | 4 (3.1) | 3 (3.0) | 2 (1.7) | 3 (1.6) | 0 | 0 | 42 (11.9) |
| A/ O | 23 (18.0) | 21 (20.8) | 40 (33.3) | 60 (31.9) | 54 (32.1) | 237 (43.0) | 122 (34.6) |
| B/ B | 0 | 0 | 0 | 0 | 0 | 0 | 10 (2.8) |
| C/ C | 4 (3.1) | 4 (4.0) | 0 | 2 (1.06) | 5 (3.0) | 56 (10.2) | 0 |
| D/ D | 0 | 0 | 0 | 0 | 0 | 0 | 7 (2.0) |
| B/ C | 0 | 0 | 0 | 2 (1.06) | 0 | 1 (0.2) | 1 (0.3) |
| C/ D | 2 (1.6) | 2 (2.0) | 0 | 0 | 0 | 0 | 0 |
| O/ O | 6 (4.7) | 6 (6.0) | 0 | 4 (2.1) | 5 (3.0) | 57 (10.4) | 18 (5.1) |

Table 3.16 MBL structural and promoter polymorphisms in the studied cohort and other African populations

| MBL Genotype | Studied group (All) n=128 (%) | Studied group (African) n= 101 (%) | Ghanaians n= 551(%) | Mozambique n= 154 (%) |
|---------------------|--------------------------------------|---|----------------------------|------------------------------|
| YA/ YA | 64 (50) | 47 (46.5) | 171 (31) | 66 (42.86) |
| YA/ XA | 33 (25.8) | 26 (25.7) | 81 (14.7) | 21 (13.64) |
| XA/ XA | 2 (1.6) | 1 (1.0) | 6 (1.1) | 2 (1.29) |
| YA/ Y0 | 18 (14.1) | 17 (16.8) | 189 (34.3) | 41 (26.62) |
| XA/ Y0 | 5 (3.9) | 4 (4.0) | 47 (8.6) | 14 (9.09) |
| Y0/ Y0 | 6 (4.7) | 6 (5.9) | 57(10.4) | 10 (6.49) |

Additional comparisons between different African populations and Oxford control group were carried out (Roy *et al.*, 2002). There was a highly significant difference in WT MBL gene distribution between Ghanaians and the Oxford population, ($p < 0.0001$). The frequency is 257/551 (46.6%) for Ghanaians whilst this is higher 213/353 (60.3%) for Oxford controls. There is also evidence of a difference in MBL gen A/O genotype distribution between these studies populations, ($p=0.01$). The frequency is 237/551 (43%) for Ghanaians while this is lower 122/353 (34.6%) for the Oxford population. When compared differences in MBL genotype O/O between Ghanaians and Oxford significant difference was also found, ($p < 0.01$). The frequency is 57/551 (10.3%) for Ghanaians while this is lower 18/353 (5.1%) for Oxford control. Other relations between studied African and Oxford populations were considered. These are not of statistical significance such as comparing Gabonese or Soweto, South Africans with Oxford population.

Studied population of African HIV-infected children ($n=101$) was compared with other African cohorts and Oxford control. There is some evidence of difference in MBL genotype A/O distribution in our cohort of patients and Africans. There is a difference between our cohort and Gabonese HIV uninfected population, ($p=0.04$). The frequency is 21/101 (20.8%) for our cohort while this is higher 40/120 (33.3%) for Gabonese. Similarly, there is some evidence in MBL A/O genotype distribution between our cohort and Soweto, South African, ($p=0.04$). The frequency is higher 54/168 (32.1%) for Soweto, South Africa. We looked at other relations in WT and MBL homozygous genotype distribution and no significant difference was found. Interestingly, there is

strong evidence of difference in MBL WT and genotype A/ O distribution between our studied cohort and Ghanaians ($p < 0.0001$), and Oxford ($p = 0.01$).

The WT (A/A) frequency is 74/101 (73.3%) for our cohort while is lower 257/551 (46.6%) for Ghanaians and 213/353 (60.3%) for Oxford. The A/O genotype distribution varies significantly between our studied African group and Ghanaians and Oxford control. The frequency is 21/101 (20.8%) for our cohort while this is higher 237/551 (43%) for Ghanaians and 122/352 (34.6%) for Oxford control. There was no significant difference in MBL O/O genotype distribution between our cohort of patients and Ghanaians or Oxford.

The additional effect of X/Y polymorphism in MBL gene (Table 3.16) on HIV status in our cohort of HIV infected children has been compared with other African population with known MBL gene polymorphisms including X/ Y genotype. There is difference in MBL genotypes: YA/ YA, YA/ XA, and YO/YO distribution between studied HIV infected children and Ghanaians ($p < 0.01$). The YA/ YA MBL genotype frequency is 47/101 (46.5%) for studied group while this is lower 171/551 (31%) for Ghanaians. The YA/XA MBL genotype frequency is 26/101 (25.7%) for studied group while this is lower 81/551 (14.7%) for Ghanaians. The frequency for YA/ YO genotype in studied group is 17/101 (16.8%) while this is higher 189/551 (34.3%) for Ghanaians. Similar relations were studied between our cohort of HIV infected children and Mozambique population. Amongst studied MBL genotypes, there only significant difference was observed within MBL genotype YA/ XA, ($p = 0.01$). The frequency is lower 21/154

(13.6%) for Mozambique when compared to studied population (25.7%). Other comparisons were not of significance.

In addition, the optional control group for comparison would be an ethnically and age matched group with known HIV status who were simultaneously studied for MBL genotype and phenotype at the same time and in the same laboratory using the same comparable methods of MBL detection. The HIV status of published paediatric studies listed in Table 3.15 is unknown except for the Gabonese study and a British Caucasians, which were adults' studies. Other studies listed in Table 3.16 were all paediatric studies. The Soweto control group included infants aged 1-12 months requiring hospitalization for lower respiratory tract infection caused by respiratory syncytial virus (RSV) (n= 55) and 113 age-and-sex ethnically matched controls (Kristensen *et al.*, 2004). The second control population included children between 0 and 13 years old from Ghana (n=323) admitted with *P. falciparum* malaria to hospital and healthy children between 5 and 15 years of age with or without detectable *P. falciparum* (n=228) from a local area (Garred *et al.*, 2003). One must emphasize that the HIV status of these paediatric populations is unknown.

3.5.2 The potential role of MBL in HIV infection in children

The frequency of MBL gene mutations in our cohort was 26.8 % in total (including heterozygous and homozygous). Interestingly, MBL mutations were found to be underrepresented in our patient cohort when compared to the other cohorts (Tables 3.15, 3.16). Lack of a control ethnically matched group with known MBL genotype status is a

limitation of the present study. The explanations as to why we observed lower frequencies of MBL variant alleles in our cohort of patients are unknown. However, if confirmed, it may suggest that MBL deficiency may have a protective role in HIV infection. Furthermore, the ethnic selection in our study group may help to explain the difference in findings between the previously published paediatric studies in Africa. The majority of children in our cohort are of African origins who have survived before arrival to the UK as the median age of the studied group was 8.9 years. They had been diagnosed with HIV at different clinical stages (progression) of the disease. We suspect that some of the African children with advanced HIV disease or AIDS presumably associated with variant O allele could have life expectancy (duration of survival) shorter than those with wild-type alleles and consequently died in early life with AIDS before being diagnosed or prior to introduction of antiretroviral therapy (ART). In our Family Clinic at GOSH 41 children died between 1991 and 1996. In contrary, only 16 children died between 1997 and 2004 when ART became available. If this hypothesis is confirmed, MBL deficiency must have a negative effect on disease progression and subsequent death.

Despite impressive *in vitro* data on MBL and HIV interaction initiated since 1989 (Ezekowitz *et al.*, 1989, Haurum *et al.*, 1993, Saifuddin *et al.*, 2000, Spear *et al.*, 2003, Ying *et al.*, 2004, Marzi *et al.*, 2007), epidemiological studies have been less conclusive. There is a broad agreement that MBL deficiency, assayed by genotype or phenotype, is associated with a greater susceptibility to HIV acquisition. MBL deficiency increased the acquisition of HIV infection by 3 and 8 fold (Garred *et al.*, 1997a; Garred *et al.*, 1997 b; Nielsen *et al.*, 1995; Prohaszka *et al.*, 1997) and increased the risk of vertical

transmission from infected mothers to their offspring (Boniotto *et al.*, 2000; Boniotto *et al.*, 2003).

Few studies have investigated the impact of MBL during paediatric HIV infection. The presence of the mutated MBL allele was significantly more frequent in HIV infection in perinatally exposed Brazilian children than in unexposed uninfected children (Boniotto *et al.*, 2003). Similar findings suggesting that MBL deficiency predisposes to increased susceptibility to HIV infection in adults has also been reported (Garred *et al.*, 1997a). HIV-infected patients were more likely to be homozygous for MBL variant alleles. Another study performed by the same group in an African population suggested that MBL mutations occurred more frequently in HIV infected patients when compared to uninfected controls (Garred *et al.*, 1997b). Prohaszka *et al.*, 1997 demonstrated lower serum concentrations of MBL in HIV- seropositive patients compared with negative controls, a finding was also confirmed by Nielsen *et al.*, 1995.

Other studies, however, did not find similar associations. For example, Pastinen *et al.*, (1998) found that high MBL levels which correspond to wild-type alleles were significantly associated with acquiring HIV. Senaldi *et al.*, found MBL levels to be higher in HIV patients than controls (Senaldi *et al.*, 1995). While another study found similar MBL genotype frequencies in Colombian HIV-infected and control individuals and further no link between MBL levels and HIV transmission was established (Malik *et al.*, 2003). Interestingly, the recently published study by Garcia-Laorden and co-workers has found a decreased frequency of MBL variants in HIV infected population (Garcia-

Laorden *et al.*, 2006) which corresponds to our finding. The majority of studies were performed before the era of ART.

A clear difference in natural HIV progression between adults and children has been observed with respect to transmission, pathogenesis and clinical manifestations (Scott *et al.*, 1984; Ebrahim *et al.*, 1991). In our population study ~23 % of HIV- infected children had an MBL polymorphism and of these 69% were receiving combination antiretroviral (HAART) therapy. In contrast, only 10% HIV positive children defined as LTNP and 22.8 % ARTN exhibited MBL polymorphism. We found that proportion of MBL genotypes were not significantly different between groups on different therapeutic regimens. It is most likely that due to the lack of greater number in our cohort of LTNPs that no significant difference in MBL genotype distribution was noted between the various groups on different therapeutic regime. Unfortunately, no information on disease progression was available for the studied Brazilian HIV infected children, but the findings of Boniotto and co-workers with respect to an Italian paediatric population seem to confirm a role for MBL in HIV disease progression (Boniotto *et al.*, 2000). Other study by Amoroso and colleagues showed that children with rapidly progressing disease are more likely to have MBL alleles (codon 54) therefore MBL mutation confers a relative risk for rapid progression to AIDS (Amaroso *et al.*, 1999). Our hypothesis of normal MBL levels in LTNPs is consistent with other studies which showed no reduction in MBL serum concentration in LTNPs with HIV infection (Maas *et al.*, 1998, Hundt *et al.*, 2000). These combined results suggest that wild type MBL may be a protective host factor in HIV infection. Furthermore, the ethnic selection in our study group may help to explain difference in findings between the previously published

paediatric studies. The majority of children are of African origin who survived before arrival to the UK. We suspect that some of the African children with advanced HIV disease or AIDS presumably associated with variant O allele could have life expectancy shorter than those with wild-type alleles and consequently died in early life with AIDS before being diagnosed or treated.

In conclusion, HIV-infected children with MBL polymorphism exhibited a trend towards an increase in the rate of disease progression. The frequency of MBL genotypes were higher in children on HAART compared with LTNP or ARTN individuals. The detection of MBL mutations may be a useful predictor to identify children with delayed disease progression who, consequently, may not require immediate antiretroviral treatment.

CHAPTER 4

THE ROLE OF MBL IN SUSCEPTIBILITY TO INFECTION IN PRETERM NEONATES

4.1 INTRODUCTION

Despite advances in perinatal care, neonatal infection remains an important cause of morbidity and mortality, particularly among very low birth weight (VLBW) preterm infants (Stoll *et al.*, 2002; 2005). Reported rates of infection vary considerably, but may be higher than 20% in low gestational ages (Stoll *et al.*, 2005). It would appear that this figure has not changed dramatically in the last decade. Neonatal deaths attributed to sepsis are still considerable with mortality rates as high as 36% in Gram negative sepsis and 32% in fungal sepsis (Stoll *et al.*, 1996; Karlowicz *et al.*, 2000).

Numerous factors are responsible for the high rate of infection in this patient population. Neonates of low gestational age and birth weight are particularly at risk of infection, in part due to intensity of supportive care required, which include mechanical ventilation, parenteral feeding and the requirement for prolonged intravenous access. In addition, there are immunological reasons why infection occurs. IgG concentration in preterm neonates depends on active placental transfer and increases with gestational age (Landor 1995). The absence of passively derived maternal antibody such as that directed against Group B *Streptococcus* appears to be important (Klegerman *et al.*, 1983, Sandberg *et al.*, 2000, Luck *et al.*, 2003). Indeed premature neonates of less than 32 weeks of gestation have fetal IgG concentrations of less than 50 % of maternal levels (Christensen *et al.*, 1984; Lin *et al.*, 2001). In addition, many elements of the acquired immune system are also either low or function sub-optimally (Table 4.1). It is in these circumstances, that the innate immune system may be particularly important in providing protection against infection.

4.1.1 The potential role of MBL in early life

A number of studies have now looked at MBL in the neonatal setting (summarised in Table 4.2). Terai and co-workers reported that in term neonates, MBL levels increased during the first five days after birth (Terai *et al.*, 1993). Thiel showed that MBL levels at term were only a third of those seen at 3 months (Thiel *et al.*, 1995). It would appear from a number of subsequent publications that MBL levels are lower in preterm than term neonates and is related to gestational age rather than birth weight (Lau *et al.*, 1995; Hilgendorff *et al.*, 2005; Frakking *et al.*, 2006). It is currently unknown whether MBL levels play a clinical role in preterm neonates.

4.2 AIMS

The aims of the study presented in this chapter were:

- 1) To investigate the impact of MBL genotype/ phenotype on susceptibility to infection in preterm neonates during the first month of life.
- 2) To assess the developmental profile of MBL levels in relation to genotype, gestational age and birth weight.

| T LYMPHOCYTES and ANTIGEN PRESENTATION | | |
|---|---|--|
| Class II MHC molecule | Expression by monocytes and B cells. Variable data depending upon report. Expression either similar or reduced. | Keever <i>et al.</i> , 1995 Clerici <i>et al.</i> , 1993 |
| Interactions between T cells and Antigen-Presenting Cells (APC) | T cell surface molecules: CD2, LFA-1(CD11a-CD18) CD45R0, ICAM-1 (CD54), CD40 ligand lower on neonatal T cells. Reduced or delayed T cells responses. Reduced function of dendritic cells in neonates | Hunt <i>et al.</i> , 1994 Nelson and Holt;1995 |
| T cell cytokine responsiveness | Cytokine deficiency of neonatal T cells | Ehlers & Smith; 1991; Lewis <i>et al.</i> , 1991; Matsuda <i>et al.</i> , 1996; Garcia <i>et al.</i> , 2000 |
| T cell-mediated cytotoxicity | Neonatal T cells moderately less effective than adults T cells | Rayfield <i>et al.</i> , 1980; Harris <i>et al.</i> , 1994; Risdon <i>et al.</i> , 1994 |
| B CELLS and IMMUNOGLOBULINS | | |
| B cells | Neonatal CD5-B cells reduced expression of adhesion molecules including CD11, CD44, CD54 (ICAM-1), CD62-L(L-selectin) | Parra <i>et al.</i> , 1996 |
| Immunoglobulins | Production of Immunoglobulin by neonatal activated B cells similar to adults antigenically naive B cells or reduced Fetal B cells (mid-gestation) | Nonoyama <i>et al.</i> , 1995; Durandy 1995; |
| Natural Killer (NK) Cells | Fetal and neonatal NK cells reduced - expression of CD2, CD56, CD57 - reduced cytotoxic function - reduced cytolytic activity (NK cells , CD56-, 50 % at birth) | Merrill <i>et al.</i> , 1996 El-Sameea <i>et al.</i> , 2004 |
| PHAGOCYTES | | |

| | | |
|---|---|---|
| Neutrophils | <p>Present at birth, the numbers of circulating neutrophils increase sharply in term and preterm neonates</p> <p>Preterm neonates have limited ability to accelerate neutrophil production in response to infection-depleted neutrophil storage pool.</p> <p>Migration: adhesion of neonatal neutrophils under resting conditions is normal, whereas adhesion of activated cells is deficient (40 -50 % less)</p> <ul style="list-style-type: none"> - deficiency in L-selectin , β integrins, LFA, ICAM - chemotaxis preterm and term neonates impaired <p>Phagocytosis of bacteria by neutrophils from preterm neonates reduced</p> <p>Microbicidal activity of neonatal neutrophils decreased</p> | <p>Monroe <i>et al.</i>, 1979</p> <p>Squire <i>et al.</i>, 1979</p> <p>Christensen <i>et al.</i>, 1980</p> <p>Anderson <i>et al.</i>, 1981; 1984; 1990; 1991</p> <p>Smith <i>et al.</i>, 1990; Raghunathan <i>et al.</i>, 1982; Klein <i>et al.</i>, 1977; Falconer <i>et al.</i>, 1995; Fujiwara <i>et al.</i>, 1997; Ambruso <i>et al.</i>, 1979; Strauss <i>et al.</i>, 1980;</p> <p>Levy <i>et al.</i>, 1999; Arinola <i>et al.</i>, 2003; Koenig <i>et al.</i>, 2004</p> |
| Monocytes | <p>Delayed response (chemotaxis) in term and preterm neonates</p> <p>Capacity to produce cytokines may be reduced in term and preterm neonates</p> | <p>Sheldon and Cadwell 1963</p> <p>Hallwirth <i>et al.</i>, 2002; 2004.</p> |
| Complement | <p>Preterm neonates have a clearer and consistent decrease in both classic and alternative pathway activity.</p> <p>The levels of most complement protein increase postnatally and approach adult values by 6-18 months of age</p> | <p>Miyano <i>et al.</i>, 1991</p> <p>Arinola <i>et al.</i>, 2003</p> |
| Fibronectin | <p>Diminished levels in neonates, particularly in preterm</p> | <p>Gerdes <i>et al.</i>, 1983; Barnard and Arthur 1983, Yoder <i>et al.</i>, 1983</p> |
| C-Reactive Protein | <p>Serum levels in preterm and term neonates appear to be similar to that of adults</p> | <p>El-Sameea <i>et al.</i>, 2004</p> |
| MBL, Lipopolysaccharide-Binding Protein , Surfactant Apoproteins | <p><i>MBL is subject of this thesis and described in details.</i></p> <p>Mice deficient in SP-A are more susceptible to pulmonary infection. The role of SP-D unknown.</p> <p>Surfactant deficiency maybe one factor in the greater risk of the preterm neonate for pulmonary infections.</p> <p>LPS-binding protein, a deficiency in neonatal protein, that facilitates the response of monocytes to lipopolysaccharide</p> | <p>Cohen <i>et al.</i>, 1995</p> <p>Yoshio <i>et al.</i>, 2004</p> |

Table 4.1 Developmental Immunology of Neonate

| Study | Patients | Measurements | Assay | MBL levels (mean) µg/ml | Outcome measures |
|----------------------------------|--|---|-------|--|--|
| Terai <i>et al.</i> , 1993 | Term neonates (n=27) | MBL levels after birth and 0-5 days | ELISA | birth 1.07 5 days of life MBL=2.48 | MBL present at birth and increase in MBL levels within 5 days of life |
| Malhotra <i>et al.</i> , 1994 | MBL levels from amniotic fluid | MBL levels in relation to gestational age (26-41 wk) | ELISA | < 35 weeks of gestation MBL=0.34 > 35 weeks of gestation MBL=1.07 | significant increase in MBL levels from 32 weeks of gestation towards term |
| Thiel <i>et al.</i> , 1995 | Term neonates (n=40) Preterm neonates (n=10) | MBL levels measured during the 1 st year of life: 0, 3, 6, 9, 12 months. Preterm MBL at birth and at 10 wks of life. | ELISA | Term neonates at birth MBL=1.3 3 months MBL= 2.6 6 months MBL=2.5 9 months MBL=2.6 12 months MBL=3.1 | MBL levels increased after birth in preterm and term neonates. MBL increased in the first 10 weeks of life and further increased during the 1 st year of life. |
| Lau <i>et al.</i> , 1995 | Preterm neonates (n=168) Term neonates (n=146) | Longitudinal study MBL levels in preterm and neonates. | ELISA | Gestational age (mean) 25 weeks MBL=0.5 Postnatal age 20 weeks MBL=1.07 | MBL levels increased with gestational age. |
| Hilgendorff <i>et al.</i> , 2005 | Preterm neonates (n=47) and Term neonates (n=19) | MBL levels in relation to gestational age (24-31 wk) | | < 32 weeks of gestation MBL=0.75 Term neonates MBL=3.17 | MBL levels lower in preterm neonates <32 wks of gestation compared to term. |
| Frakking <i>et al.</i> , 2006 | Preterm neonates (n=69) Term neonates (n=16) | MBL levels measured at birth and during 3 weeks of life (n=26) | ELISA | Preterm MBL =0.98 Term MBL=1.2 | MBL levels lower in preterm neonates. Low MBL levels related to lower gestational age. MBL levels increased over 3 weeks. |

Table 4.2 List of published studies investigating the potential role of MBL in early life

4.3 PATIENTS AND METHODS

4.3.1 Patients

One hundred sixty six preterm neonates of gestational age less than < 37 weeks (age estimated from obstetrical data including last menstrual period and confirmed by ultrasound as well as by clinical examination of newborn according to the Ballard Score) were prospectively recruited for this study between September 2002 and June 2005. 67 were from the Neonatal Intensive Care Unit at University College London Hospitals (UCLH), NHS Trust, London, UK and 99 were from Polish Mother Memorial Hospital-Research Institute (PMMH-RI), Lodz, Poland. An additional cohort of 52 term neonates was also recruited to the study from Poland. Ethical approval was obtained for the study in both institutions and written parental consent was obtained prior to patient enrollment. Neonates with major congenital abnormalities or requiring surgical intervention were excluded.

4.3.2 Sample collection: MBL genotype and phenotype

All neonates included in the study had blood taken for MBL phenotyping and genotyping. Blood was collected on days 1-3, 7-10, 14-18 and 27-30 after birth and serum isolated and stored at - 80°C for future MBL analysis by ELISA (Antibody Shop/Statens Serum Institute, Copenhagen, Denmark). A total of 397 serum samples were collected longitudinally for measuring MBL protein levels, on days: 1-3 (n=130), 7-10 (n=113), 14-18 (n=91) and 27-30 (n=63). Whole blood was also obtained for MBL genotyping. Samples were collected into potassium EDTA and frozen at -80°C until

required. DNA was isolated from whole-blood samples using a commercial kit (QIAamp DNA, blood mini kit, Qiagen, Crawley, UK) according to manufacturer's instructions. In 158 preterm neonates (97 Polish and 61 British) *MBL-2* genotyping for both structural and promoter polymorphisms was successfully determined. In 8 neonates a genotyping sample was either not obtained (n=5) or was insufficient (n=3) for MBL genotyping. The genetic variants within exon 1 of the MBL gene at codon 52 (D), 54 (B) and 57 (C) were determined by polymerase chain reaction (PCR) followed by heteroduplex analysis utilising polyacrylamide gel electrophoresis. Wild type alleles were denoted as A and variant alleles as O. Promoter polymorphisms at -221 (X/ Y) of the MBL gene were also determined using a similar method. The three *MBL-2* structural gene mutations, B, C, and D are in linkage disequilibrium with the promoter region polymorphism X/Y, so that only Y associates with variant alleles.

4.3.3 Infection

Each neonate was followed up during the study period (days 0 to 30) for evidence of infection. Blood cultures were performed when infection was suspected. Definitive sepsis was diagnosed if they had the combination of a positive blood culture and clinical and/or laboratory evidence of sepsis. Clinical markers of sepsis included poor circulation (pallor, decreased perfusion, hypotension, tachycardia or bradycardia), increased oxygen requirement or ventilation parameters, temperature instability, lethargy or irritability, abdominal distension, feeding intolerance and jaundice. Laboratory markers included abnormal leukocyte count ($< 5000 \times 10^6$ cells/ L or $> 25\,000 \times 10^6$ cells/ L cells at birth, $> 30\,000 \times 10^6$ cells/ L at 12-24 hours of life, $> 21\,000 \times 10^6$ cells / L after 48

hours of life), increased immature-to-total neutrophil ratio (I/T ratio) (≥ 0.2) and / or ratio between bands and segmented neutrophils ≥ 0.3 , low platelet count ($< 150\,000 \times 10^6$ cells / L) and raised C-Reactive Protein (CRP). Positive blood cultures in the absence of clinical or laboratory evidence of sepsis were considered to be contaminants and excluded from the study. Neonates were 'presumed' to have sepsis if they had clinical or laboratory evidence of sepsis but without positive blood cultures. The clinical data was collected prospectively and a diagnosis of sepsis was recorded. However to provide a more consistent evaluation of this diagnosis, the designation of a sepsis diagnosis was made by clinicians (including Research Fellow A.D.) who reviewed the notes retrospectively. During assessment they were blinded from the MBL data. In the UK cohort the duration of antibiotic therapy used in the first 30 days of life was also recorded as a proxy marker of sepsis. This information was not recorded in the Polish cohort.

4.3.4 Methods and Statistical analysis

The detailed description of laboratory methods used for assessing MBL genotype and phenotype are presented in Chapter 2. To ease interpretation of the relationship between MBL genotypes and clinical/microbiological data, the 3 variant alleles (D=codon 52 variant, B=codon 54 variant, C=codon 57 variant) were grouped together and denoted as an O with A indicating a normal or wild type allele. Patients were classified according to their MBL exon-1 mutations as wild-type (A/A), heterozygous (A/O), or homozygous (O/O). Genotype (or allele) frequencies were analysed by the Chi-square test. Differences between groups (MBL serum levels) were compared using the Mann-

Whitney U Test (for two groups) or Kruskal-Wallis test (three or more groups). Changes in MBL levels were analysed using a mixed effect model that took into account correlations between repeated measurements within an individual (Laird and Ware 1982). All models included an intercept for measurements performed on days 1-3, a first slope for the change to 7-10 days and then a second slope for the change from 7-10 days to the end follow-up. Then, the effect of the genotype, birth weight and gestational age at birth on baseline and evaluation of MBL levels were tested. In addition, the modification of the effects of birth weight or gestational age at birth due to genotype was checked through interaction terms. The analyses of the risk of sepsis according to MBL levels, birth weight and gestational age at birth were performed using logistic regression. Probability values were two-sided, and statistical significance was defined as $p < 0.05$. Analyses were performed with SPSS version 12.0 (SPSS, Inc, Chicago, IL, USA) and with SAS version 9.1 (SAS®, Cary, NC, USA).

4.4 RESULTS

4.4.1 Patients Characteristics

166 premature neonates (99 Polish and 67 British) were enrolled into the study. 158 were included for further analysis (see below). Within this group, the median gestational age was 30 weeks (range 24-36 weeks) and the median birth weight was 1399 grams (range 510-2958 grams). 95 out of 158 (60%) neonates had a birth weight ≤ 1500 g and were classified as very low birth weight (VLBW) and 45 (28%) had a birth weight ≤ 1000 g (extremely low birth weight, ELBW). 46 out of 158 (29%) were neonates of

gestational age ≤ 28 weeks, and 81 % of these were ELBW. There were no significant differences between the British and Polish cohorts in terms of sex ($p=0.93$), gestational age ($p=0.39$) and birth weight ($p=0.37$).

The detailed demographic characteristics are shown in Table 4.3.

Table 4.3 Demographic characteristics of preterm neonates in this cohort (n=158)

| Characteristics | British neonates n=61 | Polish neonates n=97 | Total n (%) |
|----------------------------|----------------------------------|---------------------------------|------------------------|
| Ethnicity | | | |
| White | 27 | 97 | 124 (79) |
| Black | 7 | - | 7 (4) |
| White& Black | 7 | - | 7 (4) |
| Asian | 8 | - | 8 (5) |
| Others/unknown | 12 | - | 12 (8) |
| Sex | | | |
| Female | 27 | 44 | 71 (45) |
| Male | 34 | 53 | 87 (55) |
| Gestational age, wk | | | |
| ≤ 28 | 23 | 23 | 46 (29) |
| 29- 30 | 7 | 25 | 32 (20) |
| > 30 | 31 | 49 | 80 (51) |
| Birth weight, g | | | |
| ≤ 1000 (ELBW) | 23 | 22 | 45 (28) |
| ≤ 1500 (VLBW) * | 37 | 58 | 95 (60) |
| > 1500 | 24 | 39 | 63 (72) |

* including neonates with ELBW (n=45)

4.4.2 Analysis of MBL genotype

MBL-2 genotypes were successfully determined in 158 (97 Polish and 61 British) preterm neonates and 52 term neonates. Table 4.4 shows the frequency of MBL genotypes for the British and Polish neonates. There was no significant difference in the frequency of MBL genotypes between the two populations ($p=0.44$). The distribution of MBL genotypes was similar between neonates of different gestational age and birth weight (data not shown).

Table 4.4 Frequency of MBL exon-1 mutations

| MBL Genotype | Preterm neonates (n=158) | | Term neonates (n, %) |
|-----------------------------|--------------------------|-----------------|-------------------------|
| | British (n, %) | Polish (n, %) | |
| Wild type (A/ A) | 36 (59.0) | 67 (69.0) | 34 (65.0) |
| Heterozygous (A/ O) | 23 (38.0) | 27 (28) | 18 (35.0) |
| A/ B | 16 | 19 | 13 |
| A/ C | 6 | 3 | 0 |
| A/ D | 1 | 5 | 5 |
| Homozygous (O/ O) | 2 (3.0) | 3 (3) | 0 |
| B/ B | 0 | 3 | 0 |
| B/ C | 2 | 0 | 0 |
| Total | 61 (100) | 97 (100) | 52 (100) |

In addition, neonates were analyzed according to their MBL gene polymorphism in the promoter region at position -221 (X/ Y). The results are presented in Table 4.5.

Table 4.5 Frequency of MBL exon-1 and X/ Y promoter polymorphisms

| MBL Genotype | Preterm neonates (n=158) | | Term neonates (n, %) |
|---------------|--------------------------|---------------|-------------------------|
| | British (n, %) | Polish (n, %) | |
| YA/ YA | 22 (36.1) | 34 (35.1) | 14 (35.9) |
| YA/ XA | 14 (23.0) | 29 (29.9) | 9 (23.1) |
| XA/ XA | 0 | 4 (4.1) | 2 (5.1) |
| YA/ YO | 17 (27.8) | 22 (22.6) | 10 (25.6) |
| XA/ YO | 6 (9.8) | 5 (5.2) | 4 (10.3) |
| YO/ YO | 2 (3.3) | 3 (3.1) | 0 |
| Total | 61 (100) | 97 (100) | 39 (100) |

4.4.3 MBL genotype and sepsis

Forty seven neonates from the 158 with known MBL genotypes (29.7 %) had a single positive blood culture during the study period which was associated with clinical or laboratory evidence of sepsis (definitive sepsis): 16 (26.2 %) were from the British cohort and 31 (31.9 %) were from the Polish population. Nine neonates had a second positive blood culture with *Coagulase negative staphylococci* (CONS) n=3; *Enterobacter* sp. n=1, *Klebsiella* sp. n=2, *Candida albicans* n=3. These were not included in the analyses. The organisms isolated during the first episode of sepsis are

presented in Table 4.6. In both populations the majority of positive blood cultures were caused by *CONS*. Only five blood cultures were positive for Gram-negative organisms and one for *Candida albicans*. No significant association was found between definitive sepsis and MBL genotype for the neonatal population as a whole ($p=0.46$).

Table 4.6 Microorganisms associated with the first episode of sepsis.

The table shows the number of individual neonates in the studied cohort with positive blood cultures.

| Organisms | British | Polish | Total |
|--|----------------|---------------|--------------|
| Gram-positive | 15 | 26 | 41 |
| Coagulase negative <i>Staphylococcus</i> | 13 | 22 | 35 |
| <i>Staphylococcus aureus</i> | 1 | 3 | 4 |
| <i>Group B Streptococcus</i> | 1 | 1 | 2 |
| Gram-negative | 1 | 4 | 5 |
| <i>Escherichia coli</i> | 0 | 1 | 1 |
| <i>Klebsiella sp.</i> | 1 | 0 | 1 |
| <i>Enterobacter sp.</i> | 0 | 1 | 1 |
| <i>Haemophilus influenzae</i> | 0 | 1 | 1 |
| <i>Corynebacterium macginleyi</i> | 0 | 1 | 1 |
| Fungi | 0 | 1 | 1 |
| <i>Candida albicans</i> | 0 | 1 | 1 |
| Total | 16 | 31 | 47 |

Of the 47 positive blood cultures, 42 were from VLBW neonates. Of these, 17 had MBL variant alleles and 25 were WT. There were 53% (17/32) of the VLBW neonates with variant alleles who had a definitive sepsis compared to 39% (25/63) in the WT VLBW population and this just failed to reach significance ($p=0.053$). Analyses of the effect of MBL genotype, gestational age ≤ 28 weeks and birth weight ≤ 1000 g also did not show a statistically significant association with sepsis. Interestingly, 4 of the 5 patients who were found to be homozygous for MBL variant alleles had a definitive sepsis. There was no apparent relationship between MBL genotype and susceptibility to any specific micro-organism.

In total, 80 out of 158 (50.7 %) preterm neonates were diagnosed with sepsis. Of these, 47 had definitive sepsis and 33 had presumed sepsis. 54.6% of neonates with *MBL-2* variants had sepsis compared to 48.6% with a wild type genotype (A/A), (Table 4.7). This was not significant ($p=0.47$, calculated by Chi-square).

Table 4.7 MBL genotype and rate of sepsis in preterm neonates (n=158)

| MBL genotype-exon1 | Sepsis (n, %) | No sepsis (n, %) | Total |
|---------------------------|----------------------|-------------------------|--------------|
| A/ A | 50 (48.6) | 53 (51.4) | 103 (100) |
| A/ O and O/ O | 30 (54.6) | 25 (45.4) | 55 (100) |
| Total | 80 (50.7) | 78 (49.3) | 158 (100) |

Of 95 neonates classified as VLBW, 69 (72.6%) had a septic episode, definitive or presumed. 27 out of 32 (84.3%) neonates with variant alleles had a septic episode compared to 42 out of 63 (66.7%) with a wild type genotype. This just failed to reach significance ($p=0.056$), Table 4.8. Analyses of the effect of MBL genotype, gestational age ≤ 28 weeks and birth weight ≤ 1000 g also did not show a statistically significant association with sepsis, definitive or presumed.

Table 4.8 MBL genotype and sepsis in VLBW preterm neonates (n= 95); ($p=0.056$).

| MBL genotype-exon 1 | Sepsis (n, %) | No sepsis (n, %) | Total (n, %) |
|---------------------|---------------|------------------|--------------|
| A/ A | 42 (66.7) | 21 (33.3) | 63 (100) |
| A/ O and O/ O | 27 (84.3) | 5 (15.7) | 32 (100) |
| Total | 69 (72.6) | 26 (27.4) | 95 (100) |

4.4.4 MBL Phenotypic analysis

MBL levels at birth and throughout the 30 day study period were measured and related to *MBL-2* genotype, gestational age and birth weight. 130 neonates had a sample taken within the first 3 days of life and 38 neonates had 4 samples collected (completed serial measurements). As expected, serum MBL levels were significantly related to *MBL-2* genotype ($p < 0.001$). MBL levels were found to be significantly lower in neonates born at 30 weeks or less compared to term neonates ($p<0.05$), (Table 4.9).

Table 4.9 MBL levels in relation to gestational age.

Matching MBL levels and genotypes from preterm (n=130) and term neonates (n=52) analysed from samples taken between 1 and 3 days after birth.

| MBL- exon 1 Genotype | MBL levels (1-3 days of life) | Gestation ≤ 30 weeks | Gestation 31-36 weeks | Gestation ≥ 37 weeks |
|---------------------------------|--|---------------------------------|----------------------------------|---------------------------------|
| A/A | Median (ng/ml) | 2230 | 2473 | 2799 |
| | Range | 423-5097 | 1023-4494 | 1288-5386 |
| A/O and O/O | Median (ng/ml) | 182 | 341 | 511 |
| | Range | 28-816 | 17-940 | 124-793 |

MBL levels were also determined by postnatal age. Levels increased between the 1-3 day sample and the 7-10 day sample (Figures 4.1 and 4.2). The increase between the first two samples was larger in WT neonates (+243 ng/ml/day 95% Confidence Interval [CI] =+196; +291) than in neonates with MBL mutations (+43 ng/ml/day [CI] =-17; +103, $p < 0.001$). This increase remained stable for the subsequent 2 samples.

In WT neonates, baseline MBL levels were significantly lower in babies born at 28 weeks of gestational age or less (2077 vs. 2627 ng/ml, $p=0.022$) whereas they were not statistically significant in neonates with MBL mutations (214 vs. 440 ng/ml, $p=0.43$). Interestingly, the rate of change of MBL levels between the first and subsequent samples was marginally influenced by gestational age at birth during the first week, tending to be larger in neonates with lower gestational age (+82 ng/ml/day $p=0.12$). MBL levels in

WT neonates born at less than 28 weeks therefore reached those born at greater than 28 weeks at 4 weeks postpartum (3900 vs. 3956 ng/ml, $p=0.84$).

The difference in baseline MBL level between neonates with very low birth weight (≤ 1500 g) and the others were not significant either in WT ($p=0.73$) or neonates with variant alleles ($p=0.57$). However in WT neonates baseline MBL levels were significantly lower in neonates with a birth weight ≤ 1000 g (2099 vs. 2654 ng/ml, $p=0.015$), or with gestational age ≤ 28 weeks. This effect was not observed in neonates with MBL mutations (216 vs. 441 ng/ml, $p=0.43$). As with gestational age, the rate of change of MBL levels between the first and subsequent samples was marginally influenced by birth weight (+62 ng/ml/d, $p=0.23$).

The independent effects of each variable were not distinguishable because birth weight and gestational age at birth were too highly correlated ($r=0.83$, $p<0.0001$).

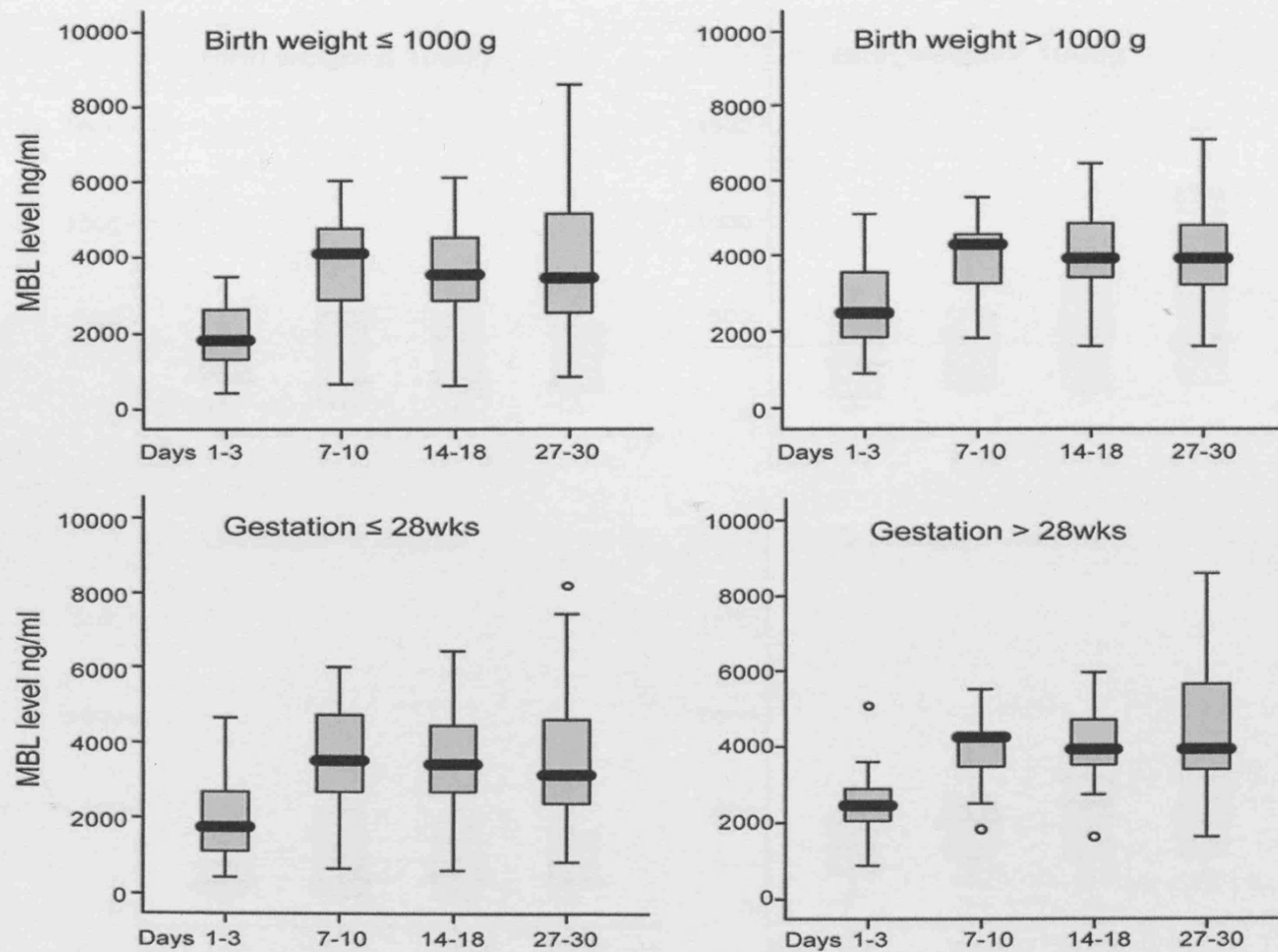


Figure 4.1: MBL levels in neonates with a wild-type MBL genotype in relation to postnatal age. The box plots show MBL levels (ng/ml) for gestational ages (≤ 28 weeks and > 28 weeks) and birth weight (≤ 1000 g and > 1000 g). The first sample was significantly lower than the three subsequent samples for all gestational ages and birth weights ($p=0.001$). Outliers are shown as black dots.

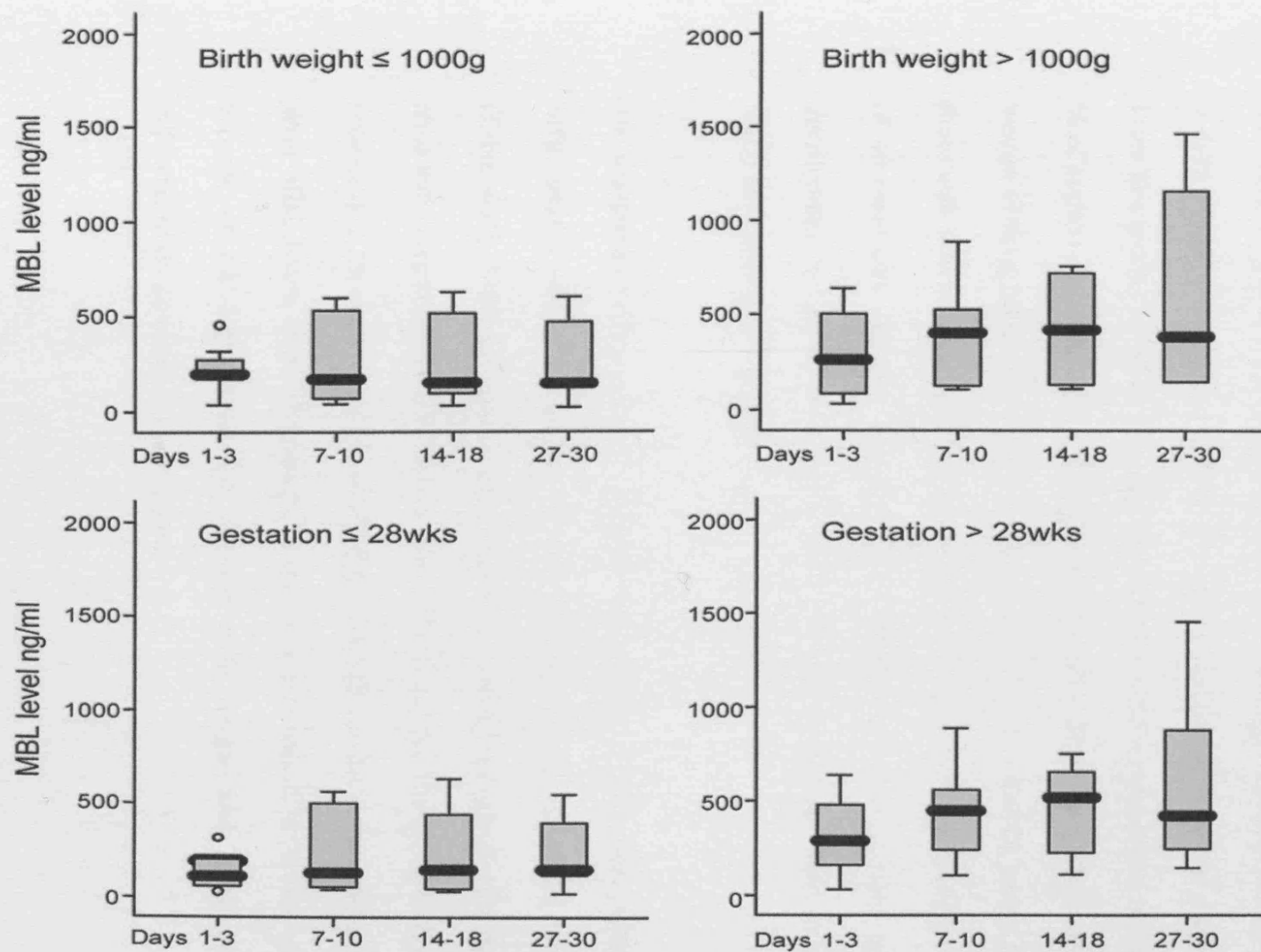


Figure 4.2 MBL levels in neonates with MBL variant alleles in relation to postnatal age. The box plots show MBL levels (ng/ml) for gestational ages (≤ 28 weeks and > 28 weeks) and birth weight ($\leq 1000g$ and $> 1000g$). The first sample was significantly lower than the three subsequent samples for all gestational ages and birth weights ($p=0.01$).

4.4.5 Risk of sepsis in relation to MBL phenotype, birth weight and gestational age

One hundred twenty neonates with known MBL genotype and MBL levels from the first 3 days of life were analyzed further to assess the risk of sepsis in preterm neonates. From this group, 37 (31%) had a definitive sepsis (confirmed positive blood culture). 45 % of positive blood cultures occurred within the first week of life. Neonates with a birth weight ≤ 1000 g had a higher risk of sepsis (54% vs. 22%, OR=4.2, $p < 0.0001$) as well as those with ≤ 28 wks of gestational age at birth (55% vs. 23%, OR=4.0, $p < 0.001$). 52% of neonates with an MBL level at birth of ≤ 400 ng/ml ($n=27$) (first quartile of the distribution), had sepsis compared to 26%, OR=3.1, $p=0.01$ in neonates with an MBL levels of greater than 400ng/ml.

The independent effect of birth weight, gestational age at birth when adjusting for low MBL level (≤ 400 ng/ml) through a multivariable logistic regression was performed (Table 4.10). MBL level and birth weight (≤ 1000 g) and gestational age (≤ 28 wks) remained independently associated with the risk of sepsis. The probability of sepsis in a neonate of ≤ 28 weeks GA or ≤ 1000 g BW with MBL levels \leq than 400ng/ml was 70%. With MBL levels above 400ng/ml the risk of sepsis was 47% in both groups. Again because of the high correlation between birth weight and gestational age their independent effect could not be checked.

Table 4.10 Risk of definitive sepsis depending on MBL genotype, phenotype, gestational age (GA) and birth weight (BW). Analysis performed by Uni- and Multivariable analysis. Odds ration (OD) and p value.

| Factor | Patients (n=120) | Definitive sepsis (%) | Unadjusted effect | Multivariable model 1 | Multivariable model 2 |
|-------------------|-----------------------------|--------------------------------------|------------------------------|----------------------------------|----------------------------------|
| MBL | | | | | |
| Genotype | | | (p=0.33) | - | - |
| A/A | 77 | 29 % | 1 | | |
| A/O and O/O | 43 | 37 % | 1.48 | | |
| MBL levels | | | | | |
| | | | (p=0.01) | (p=0.03) | (p=0.04) |
| >400 ng/ml | 93 | 26% | 1 | 1 | 1 |
| ≤400 ng/ml | 27 | 52% | 3.1 | 2.9 | 2.7 |
| BW | | | | | |
| | | | (p=0.0006) | (p=0.001) | |
| >1000g | 83 | 22% | 1 | 1 | - |
| ≤1000 g | 37 | 54% | 4.2 | 4.1 | |
| GA | | | | | |
| | | | (p<0.001) | | (p<0.003) |
| >28 wks | 87 | 23% | 1 | - | 1 |
| ≤28 wks | 33 | 55% | 4.0 | | 3.7 |

In table 4.10 two separate multiple logistic regression models were presented, model 1 adjusted for birth weight (BW) and the model 2 adjusted for gestational age (GA). These two variables BW and GA were strongly correlated leading to collinearity issue when adjusting for both in the same model.

The MBL cut-off of 400 ng/ml (first quartile of the distribution), threshold of 400 ng/ml was defined a priori according to biological/ clinical hypotheses. First, in the a study of

MBL levels and genotypes from over 500 children from the AVON longitudinal study of parents and children, individuals who were either YO/YO or XA/YB or XA/YC all had levels of less than 400ng/ml (unpublished data). Second, in previous studies on MBL it was found that MBL levels below 400ng/ml did not activate complement binding and did not enhance MBL-mediated opsonophagocytosis (Neth et al., 2002).

4.4.6 MBL status and duration of antibiotic therapy

In the British neonates, the duration of antibiotic therapy used in the first 30 days of life was recorded. 47 neonates had both MBL genotype and phenotype measured in the first 3 days of life. Nineteen neonates received more than 10 days of antibiotics. Of these, 10 had one or more variant alleles. Of the 28 who received antibiotics for 10 days or less, only 9 had A/O or O/O genotypes ($p=0.08$). When antibiotic duration was related to MBL status, as determined by phenotype (Figure 4.3), median MBL levels were found to be significantly lower in patients treated with antibiotics for more than 10 days as compared to neonates receiving antibiotics for 10 days or less (422 ng/ml vs 1618 ng/ml, $p=0.02$), (Figure 4.3). Three of the 9 neonates who had a WT genotype and who received more than 10 days of antibiotics had levels of less than 1000 ng/ml. This was double the proportion (3 out of 19) of WT neonates with MBL levels of 1000ng/ml or less and who received antibiotics for less than 10 days.

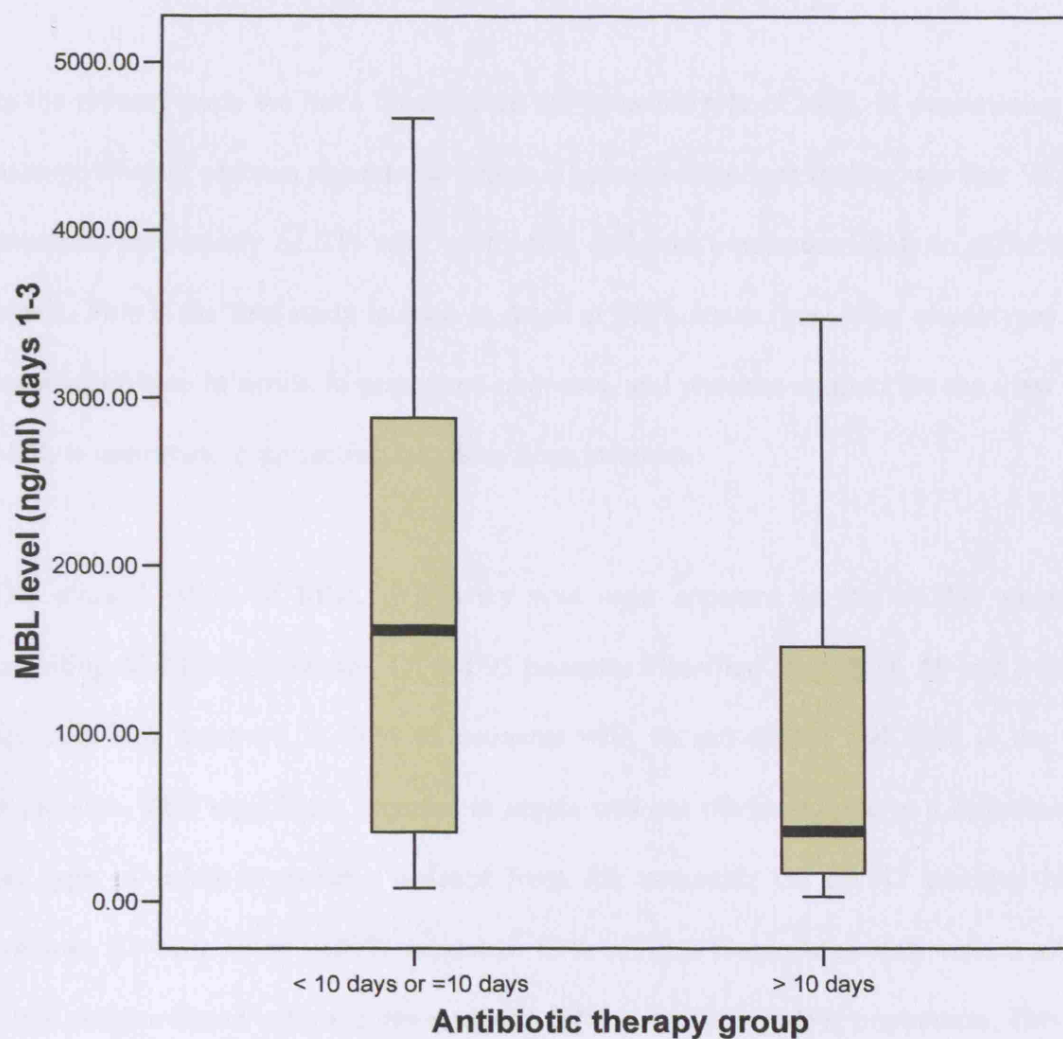


Figure 4.3 MBL levels in therapy groups (cut off 10 days) ($p=0.02$, Mann-Witney test). Box-whisker plots of MBL serum concentrations (ng/ml) in preterm British neonates (1-3 days of life). The boxed area represents 50% of values. The thick line inside the box represents the median value. Whiskers are 10-th and 90-th percentiles. Outliers are open circles.

4.5 DISCUSSION

In the present study we have investigated the potential role of MBL in determining the susceptibility of preterm neonates to sepsis. The most important finding was that VLBW neonates, particularly ELBW who were MBL deficient were more likely to suffer from sepsis. This is the first study to look in detail at MBL status (genotype/ phenotype) and susceptibility to infection in premature neonates, and provides support for the view that MBL is important in protecting neonates from infection.

The clinical effect of MBL deficiency was most apparent in the VLBW neonates including 45 ELBW neonates. Of the 95 neonates classified as VLBW, 69 had a septic episode. This occurred in 84% of neonates with variant alleles and 67% in the WT population. This significant increase in sepsis was not obviously due to a difference in the type of micro-organisms isolated from the neonates. Of the 47 positive blood cultures, 42 were from VLBW neonates. 53% of VLBW neonates with variant alleles had a positive blood culture compared with 39% in the WT VLBW population. This just failed to reach significance but indicates that one potential mechanism for why neonates with variant alleles have more sepsis is because they are more susceptible to bacterial invasion. Such an explanation is thought to explain why MBL deficient mice are more likely to succumb to *Staphylococcus aureus* and *Pseudomonas aeruginosa* infections (Shi *et al.*, 2004; Moller-Kristensen *et al.*, 2006). A recent paper has also found an association between low MBL levels and an increased risk of sepsis (De Benedetti *et al.*, 2007). In this study nearly half of the positive blood cultures grew *Klebsiella*

pneumoniae. In contrast, the rate of proven sepsis to non-CONS organisms in our study was low. This probably reflects differences in environment, supportive care practise and possibly in the volumes of blood sampled. However it is also possible that the difference between the WT and variant MBL populations is not just the rate of sepsis but also the host response to a bacterial challenge. In a recent study of children with systemic inflammatory response syndrome (SIRS), MBL deficiency was highly correlated with the development of SIRS (Fidler *et al.*, 2004). This was not apparently due to an increase in invasive bacterial infections. It has now been established that MBL can modulate cytokine production *in vitro* and *in vivo* (Jack *et al.*, 2001; Shi *et al.*, 2004; Moller- Kristensen *et al.*, 2006) and this may influence the response of neonates to even minor infections. Further studies are required to establish definitively why MBL deficiency predisposes preterm neonates to sepsis.

In previous studies, gestational age and birth weight were found to markedly influence MBL levels (Lau *et al.*, 1995; Hilgendorff *et al.*, 2005; Frakking *et al.*, 2006). The effect of gestational age or birth weight on MBL levels was seen only in wild type neonates. As figures 4.1 and 4.2 demonstrate, MBL levels were lower in neonates with variant alleles, low birth weight or low gestational age at birth. By far the most important influence on MBL levels was postnatal age. In the first three days of life, all neonates, including those with variant alleles, had significantly less MBL than at the three subsequent time points analysed in this study. MBL is synthesised by hepatocytes and appears to show a similar developmental pattern to that of other proteins synthesised by the liver (Manco-Johnson 2005). The significance of this finding, also highlighted by Frakking, is that even neonates with a WT genotype may be deficient in MBL at birth

(Frakking *et al.*, 2006). This would increase the number of premature neonates who are phenotypically MBL deficient at birth above the 35% who had variant alleles. The clinical effect of low MBL levels within the WT neonates, who are in effect MBL deficient, was demonstrated in the analysis of antibiotic usage in the UK cohort. 6 of the 28 WT neonates in whom data was also available on antibiotic usage, had MBL levels of less than 1000 ng/ml. Half of these received more than 10 days of antibiotics. We consider antibiotic usage to be a marker of sepsis, as a course of at least 10 days of antibiotics was always administered for proven or presumed sepsis in the UK cohort. As such it would appear that low levels of MBL even within the WT population can predispose preterm neonates to sepsis.

The main findings of this study were that the combination of MBL deficiency and prematurity renders such neonates particularly susceptible to sepsis. The study only monitored neonatal infections throughout the first month of life. Clearly VLBW neonates may still be cared for in the neonatal intensive care unit after this time and will therefore still be susceptible to infections. Genetic MBL deficiency may still be important in this population. However, an analysis of septic episodes within the two neonatal units involved in this study shows that about half of septic episodes occurred within the first week of life. This may be at least partly due to our observation that around half of newborn babies will also be MBL deficient. MBL will become available as a therapeutic agent in the near future and from our results, may be a useful adjunct to the care of VLBW neonates (Valdimarsson *et al.*, 2003; 2004).

CHAPTER 5

GENERAL DISCUSSION

The two studies presented in this thesis provide further evidence that MBL is a determinant of infectious susceptibility and severity.

5.1 THE ROLE OF MBL IN HIV INFECTION

The results presented in chapter 3 showed that genetic MBL deficiency was found to be less common (23%) in HIV infected children in our cohort from GOSH (79 % were of Sub-Saharan origin, mean age 8.9 years) than in previously published African and UK cohorts of children and adults (Lipscombe *et al.*, 1996; Mead *et al.*, 2003; Mombo *et al.*, 2003; Garred *et al.*, 2003; Kristensen *et al.*, 2004). This finding was unexpected. From the previous literature (Lipscombe *et al.*, 1996; Garred *et al.*, 2003; Kristensen *et al.*, 2004), we expected to find an increase in MBL deficiency in the HIV population. Taken at face value, this finding could indicate that the high rate of MBL deficiency in African populations (Lipscombe *et al.*, 1996; Mead *et al.*, 2003; Mombo *et al.*, 2003; Garred *et al.*, 2003; Kristensen *et al.*, 2004) could be protective against this infection. However we think this is unlikely for two reasons. Firstly, this finding differs from previous studies in which MBL deficiency was found to be higher in HIV infected individuals. Secondly, we found a relationship between high rates of MBL deficiency in HIV patients with more severe disease, as indicated by CD4+ T cell counts, and in a small cohort of LTNP's. We conclude that MBL deficiency predisposes to HIV infection and more severe disease and as such the studied cohort had fewer MBL deficient individuals because of the death of children in the pre-HART era.

There were a number of potential problems with this study, which must be considered. Firstly, there was no ethnically matched control group. This makes interpretation of our data very difficult. Secondly, the study was cross-sectional and performed within the era of HAART. This also complicates the analyses. Thirdly, the study was performed in children and most published studies have been from adult cohorts. In spite of these potential problems interpretations of our data are consistent with the limited data published in children. MBL variant alleles were significantly more frequent in HIV infection in perinatally exposed Brazilian children than in unexposed uninfected children (Boniotto *et al.*, 2003). MBL gene polymorphisms in promoter and exon-1 region were also correlated with HIV disease progression (Amaroso *et al.*, 1999; Boniotto *et al.*, 2000) and children with rapidly progressing disease were more likely to have MBL variant alleles (codon 54) (Amaroso *et al.*, 1999).

It is difficult to see how this data will impact on the management of children with HIV infection. It may allow for the identification of individuals who may not cope well with ART interruption or even moderate disease. It is unlikely that MBL would be useful therapeutically. This is because it would probably have to be given very frequently as the MBL half life is short and also MBL appears to have limited antiviral activity in the physiological doses ranges (Ying *et al.*, 2004).

5.1.1 MBL and other viral infections

MBL has been shown to bind to a number of viruses including Influenza, Hepatitis B and C and SARS (Thielens *et al.*, 2002). However as with HIV binding will not always indicate a role in the clinical settings. The role of MBL in chronic hepatitis B and C viral infections has been investigated in adults' populations (Thomas *et al.*, 1996; Yuen *et al.*, 1999; Sasaki *et al.*, 2000; Kilpatrick *et al.*, 2003; Thio *et al.*, 2005). An association of exon-1 MBL gene with persistent HBV infection and progression of HBV and HCV disease has been found (Thomas *et al.*, 1996; Yuen *et al.*, 1999, Sasaki *et al.*, 2000). Several studies have suggested that low-MBL producing genotypes are associated with a poor response to interferon treatment in chronic hepatitis C patients (Matsushita *et al.*, 1998; Sasaki *et al.*, 2000).

5.2 THE ROLE OF MBL IN NEONATAL INFECTION

Several genes have been examined to ascertain if they play a role in the inflammatory response and in sepsis. These include the genes for tumor necrosis factor (TNF)-alpha, TNF-beta, the interleukin (IL)-1 receptor antagonist, heat shock protein (hsp70), IL-6, IL-10, CD-14, Toll-like receptors: TLR-2, TLR-4 , and NOD2 (Weitkamp *et al.*, 2000; Holmes *et al.*, 2003; Levy *et al.*, 2004; Ahrens *et al.*, 2004). However, to date only one such study has been performed in preterm neonates. 356 VLBW neonates were genotyped for MBL variants, the IL-6-174 G/C polymorphism, CD14-159 T mutation, TLR4-896G mutation and NOD2-3020 ins C-mutation. Sepsis in VLBW infants was

defined as blood culture-proven sepsis (Ahrens *et al.*, 2004). VLBW neonates who did not carry any of the mutations had a lower frequency of blood culture proven sepsis than neonates who did carry mutations. Carriers of the homozygous IL-6-174 G mutation and carriers of the NOD2-3020ins C mutation had a higher rate of sepsis than non carriers. Within, the limits of the study size (n=50), the MBL B/C/D alleles analyzed did not appear to substantially influence the risk of neonatal sepsis (Ahrens *et al.*, 2004).

The study presented in this thesis differed from the Ahrens study described above. Information on both MBL genotype and phenotype were obtained as well as the rates of sepsis in preterm neonates within a wide range of gestational age (24-36 weeks of gestation) and birth weight (510-2958 grams). In addition the study included almost three times as many patients as the Ahrens study.

The gene frequencies of *MBL-2* gene polymorphisms in exon-1 region (both heterozygous and homozygous) investigated in our cohort of neonates (41 % in the UK cohort; 33 % in the Polish cohort) were in keeping with the frequencies previously published in other UK cohorts (Mead *et al.*, 1997; Roy *et al.*, 2002). There was no obvious association between MBL genotype and risk of prematurity. The presence of polymorphisms in the *MBL-2* gene appeared to be associated with the development of sepsis in VLBW neonates. 84 % of genetically MBL deficient VLBW neonates were septic (blood culture proven and clinically presumed sepsis) compared to 66% septic neonates with a wild-type *MBL-2* genotype. Low MBL levels through prematurity, and not only through *MBL* variant alleles, also contributed to the population of neonates who were MBL deficient. This was particularly marked in ELBW neonates and or gestation

of ≤ 28 weeks. These findings were in accordance with the two recently published studies (Hilgendorff *et al.*, 2005; Frakking *et al.*, 2006).

MBL deficiency as detected by genotype and phenotype predisposed VLBW neonates to sepsis. The risk of infection was particularly high (45%) in the first week of life. This corresponds to the period in which MBL levels were at their lowest. Development has been shown to be a significant influence on the production of components of the MBL-lectin and associated pathways. MBL appear to show a similar developmental pattern to that of other proteins synthesized by the liver (Manco-Johnson, 2005; Nava *et al.*, 2005). MASP-1 and MASP-2 mRNA was expressed as early as embryonic day 12.5. Its expression progressively increased during development, peaked around birth and slightly declined in adult stages. The expression profiles of Ficolin A in the embryo and liver closely resembled that of the MASPs (Liu *et al.*, 2005).

Further studies are required to establish the significance of MBL deficiency in predisposing neonates to infection. Further studies are also required to explore the impact of the entire MBL-lectin pathway including its associated serine proteases, MASPs. Recently, a genetically determined deficiency of MASP-2 was described (Stengaard-Pedersen *et al.*, 2003). The mutation of a single nucleotide leads to an ASP-Gly exchange in CUB 1 domain that renders MASP-2 incapable of binding to MBL. The consequences of MASP-2 deficiency might be more severe since MASP-2 mediates the activation of complement through MBL. Therefore it would be especially relevant to determine whether low serum levels of MASP-2 correlate with susceptibility to sepsis in neonates. This analysis is ongoing and the results will be known in near future.

The presence of MBL variant alleles is known to be associated with the development of sepsis in adults with systemic inflammatory response syndrome (SIRS; Garred *et al.*, 2003). A paediatric study implicating MBL deficiency with increased incidence and severity of SIRS in MBL-deficient subjects has also been reported (Fidler *et al.*, 2004).

Although the pathophysiology of SIRS and sepsis is very complex, it has been shown that genetic factors of importance for the inflammatory response such as polymorphisms associated with regulation of the expression of TNF- α and IL-1 receptor antagonist gene may be associated with susceptibility to and outcome of sepsis (Mira *et al.*, 1999; Arnalich *et al.*, 2002). It has now been established that MBL can modulate cytokine production and this may influence the response to infection (Jack *et al.*, 2001). Moreover, genetic complement deficiency and complement depletion have been shown to be beneficial in animal models of complement dependent inflammation (Czermak *et al.*, 1999). Thus, complement and, probably MBL may play different roles during the course of sepsis and systemic inflammatory response syndrome (SIRS). The factors that modulate and regulate MBL levels remain unclear and require further research.

Future studies investigating other genes involved in innate immunity such as: TLRs, NOD-2, Interleukin-6 (IL-6), and Defensins should provide a more comprehensive understanding of the role of these genes in the susceptibility and outcome of sepsis in preterm neonates. The application of molecular methods to identify neonates with genetic variants (Kilpinen *et al.*, 2001; Gibot *et al.*, 2002; Lorenz *et al.*, 2002) would help to identify which neonates are at particular risk of sepsis and may benefit from adjunctive /novel therapies.

From our results it would appear that MBL can protect VLBW neonates from sepsis. The potential therapeutic use of supplementing low MBL levels either by plasma-derived or recombinant protein in various clinical conditions such as VLBW preterm neonates and during sepsis may provide a promising approach towards decreasing mortality and morbidity in this population (Valdimarsson *et al.*, 1998; Vorup-Jensen *et al.*, 2001).

In summary, the results presented in this thesis provide support for the view that MBL plays a role in neonatal infection and in paediatric HIV disease. Further work is needed before the clinical significance of these findings is known and before therapeutic use of MBL in these populations of patients can be investigated.

PUBLICATIONS:

Dzwonek A, Novelli V, Bajaj-Elliott M, Turner M, Clapson M, Klein N. Mannose- binding lectin in susceptibility and progression of HIV-1 infection in children. *Antiviral Therapy*, 2006; 11 (4): 449-505.

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Agnieszka B. Dzwonek, Olaf Neth, Ewa Gulczynska, Marcia Chilton, Jane Hawdon, Henryk Tchorzewski, Nigel J. Klein. The role of Mannose binding lectin (MBL) in susceptibility to infection in preterm neonates. *Pediatric Research* (submitted)

PRESENTATIONS at National & International Conferences / Meetings

ORAL:

Mannose-binding lectin: its role in paediatric HIV infection. 10th Spring Meeting, RCPCH, April 2006, University of York.

Mannose binding lectin (MBL) and susceptibility to infection in premature neonates. 4th World Society for Paediatric Infectious Diseases, September 2005, Warsaw, Poland

British Paediatric Allergy Immunity Infection Group and Dutch Paediatric Infectious Disease Group, Dutch Paediatric Immunology Group, Joint meeting "Mannose binding lectin (MBL) in health and Disease in preterm neonates" The Netherlands (October 2004).

POSTERS (presenting author):

The potential role of Mannose binding lectin (MBL) in susceptibility and progression of HIV-1 infection in children 3rd IAS Conference on HIV Pathogenesis and Treatment, July 2005, Rio De Janeiro, Brazil

Mannose binding lectin and susceptibility to infection in pre-term neonates 14th European Congress of Clinical Microbiology and Infectious Diseases, May 2004, Prague, Czech Republic

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